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Brine-induced mortality of non-indigenous species in ballast water

Johanna Bradie
University of Windsor

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By

Johanna Bradie

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by

Johanna Newbold Bradie

APPROVED BY:

_____

Dr. Trevor Pitcher
Department of Biological Sciences

_____

Dr. Hugh MacIsaac, Co-advisor
Great Lakes Institute for Environmental Research

_____

Dr. Sarah Bailey, Co-advisor
Department of Fisheries and Oceans Canada

_____

Dr. Aaron Fisk, Chair of Defense
Great Lakes Institute for Environmental Research

July 23, 2009

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Abstract

Transoceanic vessels entering the Great Lakes are required to undergo ballast water exchange to reduce the risk of transporting non-indigenous species. Ballast water exchange effectively reduces invertebrate density and richness in ballast; however, an alternative treatment is required for non-compliant ships.

Sodium chloride brine was proposed to treat residual and incompletely-exchanged ballast water. Laboratory experiments were conducted to determine the minimum brine treatment to exterminate >95% of ballast water taxa. Invertebrate communities were exposed to a range of brine concentrations (15‰ to 115‰) until complete mortality was reached.

Biological evidence supports a one-hour exposure to 115‰ brine to treat ballast water. This treatment is broadly effective (>99.9%), regardless of treatment temperature, taxonomic group, or species' habitat salinity. A median of 0.00% (range 0.00-5.33) of individuals in ballast are expected to survive treatment, and the expected number of individuals released is within Canadian discharge standards. Before implementation, ship-scale trials are required.

Dedicated to my parents Andrew and Karen

For their loving support and consistent encouragement

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Introduction

A non-indigenous species (NIS) is a species that has established outside of its native range. NIS are the second greatest cause of species endangerment globally (Lawler *et al.*, 2006), and the greatest threat to biodiversity in freshwater ecosystems (Millenium Ecosystem Assessment, 2005). It is expected that all ecosystems will suffer severe impacts from NIS as introductions continue (United States Congressional Office, 1993). Additionally, NIS affect the economy, health and welfare of citizens (Colautti *et al.*, 2006a).

The economic impacts of NIS can be both direct and indirect. Production losses, increased maintenance costs, control programs, and lost tourism revenue are just a few examples of ways NIS can negatively impact the economy. The projected costs associated with invaders in Canada range from $13.3 to $34.5 billion/year (Colautti *et al.*, 2006a), and the costs in the United States, United Kingdom, Australia, South Africa, India, and Brazil together amount to roughly $314 billion per year (Pimental *et al.*, 2005). As such, it is clear from an ecological and economic perspective that it is necessary to stop the spread of NIS.

Invasive Species in the Great Lakes

The Great Lakes have been invaded by at least 182 NIS (Ricciardi, 2006), 59 of which have established since the completion of the St. Lawrence Seaway in 1959 (Kelly *et al.*, 2009). Approximately 58-85% of established NIS are the result of unintentional introductions (Mills *et al.*, 1993; Ricciardi, 2001), and 55-

70% of these invaders have been transported to the Great Lakes in ballast water (Holeck *et al.*, 2004; Ricciardi, 2006; NRC, 2008).

To eliminate the spread of NIS, the transport of individuals, known as propagules, to new regions must be prevented (MacIsaac *et al.*, 2002; Colautti *et al.*, 2003). Propagule pressure, a measure of the cumulative number of NIS released into a new area coupled with the number of release events (Wonham *et al.,* 2000), is directly related to the probability of establishment (Kolar and Lodge, 2001; Colautti *et al.*, 2006b). Therefore, in order to stop the establishment of new NIS in the Great Lakes, managers must eliminate or significantly reduce the incoming propagule pressure. Since ballast water is historically the most important introduction vector, it is the highest priority management need.

Ballast water

Ballast water is defined by the Canada Shipping Act as "water…taken on board a ship to control the trim, list, draught, stability and stresses of the ship, and includes the sediment settled out of the ballast water within a ship" (Canada Shipping Act, 2006). Ballast water is pumped into a ship's ballast tanks to compensate for weight lost when cargo is unloaded from a ship, and pumped out when cargo is being loaded (Jenkins, 2007).

Worldwide, shipping operations move 10 billion m^{-3} of ballast water and the biota contained within that water, annually (Rigby *et al.*, 1999). Ballast water transfer provides a mechanism for aquatic biota to be transported distances far greater than their natural dispersion capabilities (Locke *et al.*, 1993; Minton *et al.*,

2005), and significantly contributes to propagule transfer in aquatic systems (Carlton, 1985; MacIsaac *et al.*, 2002).

Each ballast tank can be classified as ballast-on-board (BOB) or no ballast-on-board (NOBOB). When a tank is full of ballast water, it is classified as a BOB tank. BOB tanks can carry a large volume of water (~8500m^3 / ship) and therefore a potentially large number of propagules into the Great Lakes (MacIsaac *et al.*, 2002). When ballast water is not needed because the ship is loaded with cargo, tanks are empty and classified as NOBOB. However, due to the structure of ballast tanks and pump outlets, even NOBOB tanks carry unpumpable residual ballast water and sediment (Colautti *et al.*, 2003). Although these tanks bring a relatively low volume of water (~46.8 m^3 / ship) and number of propagules to the Great Lakes (Duggan et al. 2005), collectively the risk posed by these tanks has been high because vessels with NOBOB tanks represent ~90% of vessel traffic entering the Great Lakes (MacIsaac *et al.*, 2002; Colautti *et al.*, 2003).

In the Great Lakes, approximately 450 ships arrive from ports outside of Canada annually. These ships bring in nearly 500,000 m^3 of foreign ballast water (Mark Minton, NBIC, pers. comm.), which may introduce millions of viable invertebrates into the Great Lakes (MacIsaac *et al.*, 2002; Duggan *et al.*, 2005). In order to protect the Great Lakes, shipping regulations have been established to decrease the risk that viable propagules will be delivered to, and establish in, the Great Lakes.

Current Ballast Water Regulations

Voluntary regulations implemented by Canada in 1989, followed by mandatory regulations implemented by the United States in 1993 (United States Coast Guard, 1993), effectively require transoceanic vessels arriving in North America to undergo ballast water exchange (BWE) at sea, or equivalent treatment. These regulations aim to reduce the risk of spreading NIS, and originally targeted only BOB ships but were expanded to include NOBOB ships in 2006 (Canada Shipping Act, 2006).

Ballast water exchange is a process in which a ship either exchanges (BOB) or flushes (NOBOB) its ballast tanks with deep ocean water. Exchanged ballast water must have a salinity of at least 30‰, and be taken on board more than 200 nautical miles from land where the depth exceeds 2000 meters (Canada Shipping Act, 2006). The aim of this practice is to discharge freshwater species residing in the ballast water and replace the water with high-salinity marine water. Freshwater species that do not get flushed out to sea should be killed by incoming high salinity water, and any species that enter the tanks during flushing should be killed due to osmotic stress when released into freshwater at the destination port (Locke *et al.*, 1991, 1993; United States Coast Guard, 1993).

BWE effectively reduces the risk of spreading invasive species, particularly between freshwater regions (Gray *et al.*, 2007; Santagata *et al.*, 2008). However, a supplementary ballast water treatment is needed because, on occasion, ships cannot perform BWE or may only be able to perform partial exchange. This can occur in conditions of poor weather when exchange may risk the safety of the ship and crew, or if there is an equipment failure that prevents

exchange (Canada Shipping Act, 2006). In fact, approximately 6.5% of ballast tanks (526 tanks) in transoceanic ships arriving in the Great Lakes between 2005 and 2007 were non-compliant with exchange regulations (Matthew Deneau, Fisheries and Oceans Canada, pers. comm.).

Current protocol states that non-compliant ships must notify the Minister of Transport and will then be instructed to either (i) retain some or all ballast water on board while in Canadian waters, (ii) exchange ballast water at a specified location, (iii) discharge ballast water at a specified location, or (iv) treat ballast water in accordance with an approved method (Canada Shipping Act, 2006). Alternatives (i), (ii), and (iii) may not be economically desirable to industry, since retaining ballast can interfere with cargo operations, and exchanging or discharging ballast at a specified location may result in delays and associated costs. As such, the option of treating ballast water in accordance with an approved method may be very attractive to ship operators.

Regulations allow for environmentally-sound alternatives to BWE that are at least as effective in removing or killing harmful aquatic taxa and pathogens as BWE itself (Jenkins, 2007). More specifically, Canadian regulations state that after treatment, ballast water must not have more than:

(i) 10 viable taxa m^{-3} ≥ 50μm in minimum dimension,

(ii) 10 viable taxa mL^{-1} < 50μm and ≥ 10 μm in minimum dimension,

(iii) one colony-forming unit (cfu) of toxicogenic *Vibrio cholera* 100 mL^{-1},

(iv) 250 cfu of *Escherichia coli* 100mL^{-1} and

(v) 100 cfu of intestinal enterococci 100mL^{-1} (Table 1; Canada Shipping Act, 2006).

These thresholds are in agreement with the IMO D-2 discharge standard that will be mandatory once the IMO Ballast Water Convention is ratified (IMO, 2004).

By 2016, BWE will be phased out and all ships will be required to have a treatment system (Environment Canada, 2007). There are 26 treatment technologies currently in development that use various mechanisms such as filtration, biocides, heat exposure, electric pulse treatment, ultraviolet rays, ultrasound, magnetic fields, deoxygenation, and antifouling coatings to eliminate ballast water taxa (NRC, 1996; Lloyd's Register, 2007; Mamlook *et al.*, 2008). In fact, many of the treatment systems combine solid-liquid separation with disinfection (Lloyd's Register, 2007). However, these treatments are still in development and testing, and as of yet, Canada has not approved any of these treatments. Until these treatment systems become available, an alternative treatment is needed for non-compliant ships, and even afterwards, a treatment will be needed for occasions when the shipboard treatment system becomes inoperable.

Brine treatment

The addition of sodium chloride (NaCl) brine has been proposed as a cost-effective treatment for management of both residual and partially exchanged ballast water (Jenkins, 2007). The alteration of the physical and chemical environment caused by the addition of brine to ballast tanks is expected to cause mortality of ballast water organisms by negatively affecting their metabolic

processes (Schlieper, 1971). Changes in salinity can alter the activity rate, volume, volume regulation, internal osmotic concentration, internal ionic content, ionic regulation, respiration rate, and oxygen requirements of organisms (Schlieper, 1971). It is therefore expected that a large change in salinity will cause a great disruption in the metabolic processes mentioned above, and cause mortality of organisms. Further, as seawater (30‰ salinity) used in BWE is effective in reducing the viability of freshwater and brackish water taxa by causing osmotic stress, NaCl brine (230‰ full-strength) is expected to be at least as effective as BWE if the final salinity of the treated ballast water is at least 30‰.

Natural salt water (i.e. marine and brackish water) consists of various cationic and anionic salts which act in antagonistic ways. This enables the physiological effects of these ions to reach a balance (Schlieper, 1971). Conversely, brine is manufactured from rock salt, and therefore does not have the same balance of ions as natural salt water. Brine has higher concentrations (>2.5x) of sodium, chloride, calcium and strontium, and much lower concentrations (<5x) of potassium and magnesium. Although some studies have shown that high calcium content can negate some negative effects of salinity alteration (Schlieper, 1971), it is expected that, overall, a high concentration of salts in an “unnatural” balance will cause mortality in aquatic taxa. In fact, studies have shown that acute tolerance to NaCl is usually lower than acute tolerance to natural or artificial seawater (Kefford *et al.*, 2004)

Brine is readily-available along the Great Lakes corridor (see Appendix 1), and could easily be applied to a ballast tank by attaching a hose from a tanker truck to the tank's sounding tube. Application *via* sounding tube is ideal because it is always accessible at dock, and would allow for brine to be applied directly to the ballast tank in a location where residuals pool once the ship has stern trim (Jenkins, 2007).

The unit cost for brine production ranges between \$20-\$60 m^{-3}, but with delivery and related costs it is expected that brine treatment would cost \$130-\$180 m^{-3}. A NOBOB ballast tank generally contains less than 10 m^3 of residual water (Jenkins, 2007), and each ship carries an average of 46.8m^3 of ballast water in total (Duggan *et al.*, 2005). If a ship entered the seaway with a ballast tank at 0‰, total treatment cost would be approximately \$5200-7200 per ship. However, the majority of these costs are associated with delivery. If this treatment is put into practice, brine suppliers could significantly decrease these costs by installing large brine storage tanks at ports and arranging brine delivery from nearby production facilities. Also, it is likely that most tanks requiring treatment would have undergone partial exchange, in which case lower quantities of brine would be needed to reach the targeted treatment salinity and costs would decrease.

Santagata *et al.* (2009) conducted species-specific trials to determine the efficacy of NaCl brine treatment. It was determined that a one hour treatment of 110‰ brine was sufficient to cause 100% mortality in 95% of the species tested. These results, however, are based solely on the analysis of 33 species, 8 of

which were specifically targeted due to their high salinity tolerance. In order to better understand the efficacy of brine treatment in practice, it is necessary to conduct trials with entire zooplankton communities (Kefford *et al.*, 2005) from different habitat salinities and at different treatment temperatures.

Evaluating NaCl brine as a ballast water treatment

In this thesis, I explore the biological efficacy of NaCl brine treatment *in vitro.* I expect that most, if not all, zooplankton will be exterminated by short-term exposure to concentrated NaCl brine. The null hypothesis is that survival in control and treatment groups will be equal. To test this hypothesis, I compare the survival of aquatic invertebrates exposed to NaCl brine treatment with control survival.

The first objective of this study is to determine the brine concentration and exposure time required to exterminate at least 95% of aquatic invertebrates that may enter the Great Lakes in ballast water. I propose that higher brine concentrations and longer brine exposure times will yield increased mortality. Alternatively, the null hypothesis is that increasing the brine concentration and/or exposure time will have no effect on survival. This will be evaluated by exposing invertebrates to different brine concentrations and exposure times to determine if a difference in survival results.

To thoroughly evaluate mortality to brine exposure, I chose to examine the brine tolerance of a variety of taxa from marine, freshwater and brackish-water habitats. This was accomplished by using individuals collected from i) exchanged BOB tanks in vessels arriving in the Great Lakes, ii) the Detroit River, and iii)

ports in the North Sea. These collection sites were chosen to include i) open-ocean marine taxa and hardy coastal taxa that have survived BWE and should be representative of taxa that would likely be introduced to the Great Lakes under current ballast water management regulations, ii) freshwater taxa that currently reside in the Great Lakes system, and iii) high-risk taxa (i.e. taxa with a wide salinity tolerance that inhabit a region that has historically been a donor of Great Lakes invaders). By testing NaCl brine treatment on taxa from a variety of environments, I can be more confident that the efficacy of brine treatment reported in my study is robust, regardless of the life history of incoming NIS.

Invertebrates from ports in the North Sea, specifically Rotterdam, Antwerp and Bremen, were used to represent "high-risk taxa" for three reasons. First, shipping traffic entering the Great Lakes is dominated by ships arriving from European ports (Ruiz and Santagata, 2007), so there is a high propagule pressure from these ports to the Great Lakes. If shipments between Great Lakes ports in the United States and Canada are excluded, European ports represented 63% (1373.0 tonnes) and 35% (937.7 tonnes) of cargo shipped to and from the Great Lakes by foreign ports in 2005 and 2006 (Statistics Canada, 2006). Specifically, most of this traffic originates from the Lower Rhine region (including Rotterdam and Antwerp), other places in the North Sea (including Bremen) and the Baltic Sea (Ricciardi and MacIsaac, 2000; Colautti *et al.*, 2003; Sax and Gaines, 2008). Second, climatic matching between the Great Lakes and North Sea ports (Table 3; Reid and Orlova, 2002) makes it probable that incoming propagules from the North Sea will be able to tolerate the Great Lakes' climate.

These individuals are, therefore, a particularly high invasion risk because if delivered in ballast they have a high probability of establishing. In fact, 73 established NIS in the Great Lakes originated from the North Sea (Grigorovich *et al.*, 2003). Third, North Sea taxa are expected to be tolerant of salinity changes because they are exposed to tidal salinity fluctuations in their natural habitat (Barnes, 1994). They are therefore thought to be a good indicator of an effective treatment, because they are likely to be able to cope with moderate salinity changes. Further, since these ports have a salinity range of 0.2-30‰ (Grigorovich *et al.*, 2003; Table 3), taxa from a variety of habitat salinities can be targeted to examine the effect of increasing habitat salinity on survival to brine exposure. I expect that taxa collected from high salinity environments will be more tolerant of brine exposure than organisms collected from low salinity environments. The null hypothesis is that invertebrates from habitats of varying salinity will have equal mortality after brine exposure. This will be tested by comparing the survival of taxa from habitats of varying salinity after exposure to the same brine treatment to determine if mortality rates are consistent.

I expect that taxa collected from ports will be healthier than taxa collected from ballast tanks, and I therefore believe that port taxa will be more resistant to brine treatment. Alternatively, mortality may be consistent for taxa collected from ports and ballast tanks. This will be tested by comparing mortality rates between port and ballast tank taxa taken from the same salinity and exposed to the same brine treatment.

The second objective of this study was to determine if temperature will alter the efficacy of NaCl brine treatment. Temperature and salinity are two of the most important physical factors affecting marine and brackish-water organisms (Kinne, 1963). Most aquatic invertebrates are essentially thermo-conformers, and an increase or decrease in temperature will alter their metabolic rate (Kinne, 1963). This may cause an increase or decrease in the capacity to osmo-regulate in hyperosmotic salinities. In this way, temperature can enlarge, shift, or narrow the salinity tolerance of an organism (Kinne, 1963; Schlieper, 1971). During the Great Lakes shipping season, taxa in ballast tanks may experience temperatures from 0-27°C (Reid and Orlova, 2002). It was necessary to conduct trials at different temperatures to ensure that an approved treatment would be equally effective throughout the shipping season. I expect that invertebrates will be more resistant to brine treatment at lower temperatures. Conversely, the null hypothesis is that survival to brine treatment will not be affected by temperature. This will be evaluated by comparing survival rates after brine exposure at two temperatures to determine if a difference exists.

The final objective of this thesis was to determine if mortality to brine treatment was consistent amongst all taxa. Ballast tanks can transport large communities of zooplankton and these zooplankton can have very different physiological tolerances. It was necessary to ensure that an approved brine treatment would be sufficiently strong to cause mortality in any invertebrate transported to the Great Lakes in a ballast tank. As such, entire zooplankton communities were used to enable a greater variety of species to be tested than in

conventional species-specific studies (Kefford *et al.*, 2005). The null hypothesis is that mortality from brine exposure will be consistent for all types of invertebrates.

Methods

The efficacy of NaCl brine treatment was assessed using entire communities of invertebrates collected from the field. A total of 17 experiments were conducted on individuals with a variety of life histories exposed to various brine concentrations (15‰, 30‰, 45‰, 60‰, 77‰, 115‰), exposure times (1h-6 days) and temperatures (11°C and 22°C). A variety of brine concentrations were examined to find the lowest effective brine concentration, in order to minimize the cost of treatment while ensuring that >95% of organisms would be exterminated. Trials were ended when all organisms appeared dead, and as such, exposure times varied between one hour and six days on account of the variation in brine tolerance of taxa in trials. Finally, exposure temperatures of 11°C and 22°C were chosen based on ballast tank temperatures during sample collection in August and December, and used to examine the effect of temperature on treatment efficacy.

Field Collection

Zooplankton was collected from the field and transferred to the lab to undergo testing. Collection sites included i) exchanged BOB tanks of five ships arriving in the Great Lakes (July to November 2007), ii) the Detroit River (August 2007, May 2008) and iii) the North Sea ports of Rotterdam, Antwerp and Bremen (July to August, 2008). Slight variations in methodology were used for invertebrates collected from these three sites, and to distinguish between

methodologies, they will be referred to as ballast tank, Detroit River, and North Sea experiments, respectively.

For ballast tank experiments, animals were collected from BOB tanks of ships arriving to the Great Lakes using vertical plankton net tows (53µm). For Detroit River experiments, freshwater taxa were collected from the Detroit River using vertical plankton net tows (53µm). A volume necessary to obtain a minimum of 1000 individuals was sampled. Filtered site water, as used herein, refers to water collected at the sampling location that has been filtered (GF/F Whatman filter, 0.7 µm pore size) to remove organisms and other organic matter. Taxa were rinsed into a 25L bucket containing unfiltered site water (ballast tank or Detroit River water, respectively), for transport to the laboratory. An extra 25L of site water was collected to be filtered and used to dilute NaCl brine to test salinities. Ambient salinity and temperature were measured at the time of collection.

For North Sea experiments, samples were collected from locations of varying salinity at the ports of Rotterdam, The Netherlands (five locations- See Figure 1), Antwerp, Belgium (three locations- see Figure 2), and Bremen, Germany (one location). These ports were chosen because they have a similar climate to the Great Lakes, a wide range of ambient salinities from which to sample, and most importantly, because they are all classified as high-risk donor ports (Colautti *et al.*, 2003; Ruiz and Santagata, 2007). A trial was also conducted with a sample from the Waal River in Nijmegen, The Netherlands. Experiments were conducted in July and August 2008 and a port map was used

to determine sampling locations that encompassed various ambient salinities. Docks and other access points were used to obtain access to the water. Zooplankton was collected using vertical plankton net tows (53µm), and the volume necessary to obtain a minimum of 1200 individuals was sampled. Site water was collected by lowering a 20L bucket into the water, and temperature and salinity at time of collection were noted. Complete collection information for ballast tank, Detroit River, and North Sea experiments is available in Table 4.

NaCl brine exposure experiments

Upon arrival to the laboratory, samples collected from warm water (18-23^{o}C; see Table 4) were stored at ambient room temperature until trials began, whereas samples collected from cold water (5-15^{o}C; see Table 4) were placed in an environmental chamber at 11^{o}C; experiments began no more than 24 hours after sample collection, and animals were not fed during this interval. Each sample was thoroughly mixed and two sub-samples were taken to estimate zooplankton density. Experiments began by filtering invertebrates through a 40µm sieve and rinsing them into a counting tray with brine at a desired salinity concentration or control (filtered site water). Five replicates were set up for each concentration and control in ballast tank and Detroit River experiments, whereas four replicates were done for North Sea experiments. The volume of filtrate was dependent on animal density (target of ≥50 individuals per replicate for ballast tank and Detroit River experiments, target of ≥100 individuals per replicate for North Sea experiments since only 4 replicates were done).

For ballast tank experiments, salinities of 60‰, 77‰, and 115‰ were used based on findings of a feasibility study (Jenkins, 2007). In one trial, a salinity of 45‰ was also tested to determine if a long exposure at a lower concentration could also be effective. The major ion constituents of stock NaCl brine (Pollard Highway Products, Harrow, ON, Canada) were determined in the metals lab at GLIER, University of Windsor. Brine of desired salinity was produced by diluting stock NaCl brine (300‰) with filtered site water. Salinity was checked using a handheld or digital refractometer. Lower salinities of 15‰, 30‰, and 60‰ were used for Detroit River experiments, since preliminary trials indicated that mortality of Detroit River taxa was high even at low brine concentrations. For North Sea experiments, only salinities of 77‰ and 115‰ were tested since survival as high as ~60% was observed in one replicate (ballast tank taxa) after one hour of 60‰ brine exposure, and because personnel were limited. Ballast tank and Detroit River studies were conducted at 11^{o}C and 22^{o}C, whereas North Sea experiments were only conducted at ambient temperature, since there was no significant difference attributed to temperature after analyzing results from ballast tank and Detroit River trials (see Results).

Invertebrate survival was assessed hourly in each replicate by viewing individuals under a Leica dissecting microscope at 10-80x magnification. Taxa that did not exhibit any movement, even in reaction to stimulation with a dissection probe, were considered dead. Due to time constraints, only live or dead counts could be taken for each tray; control groups were checked to determine the number of dead taxa in each tray, whereas treatment groups were

checked to determine the number of live taxa in each tray. When all taxa in all replicates of a given concentration appeared dead, brine exposure was ended. At this time, the individuals in each replicate were rinsed into filtered site water and allowed one hour of recovery time before survival was reassessed. Water samples from each replicate were tested to ensure that test temperature and salinity were maintained until the experiment was ended.

After the final assessment, taxa were preserved in 95% ethanol. For North Sea trials, taxa alive after the final assessment were preserved separately from individuals that did not survive brine exposure. Preserved samples were later counted in entirety and zooplankton was identified using Balcer *et al.* (1984), Koste (1984), Barnes (1994), Hayward and Ryland (1995), Johnson and Allen (2005), Bartsch (2006), and Newell and Newell (2006). All surviving taxa from North Sea experiments were identified to the lowest possible taxonomic level. Additionally, fixed-count sampling techniques were employed to subsample 100 individuals from each North Sea and Detroit River trial to identify to genus level (Barbour and Gerritsen, 1996). These identifications were used to compile a non-exhaustive list of the prevalent species in trials (Appendix 2). Taxa from ballast water experiments were identified to the lowest possible level by a taxonomic expert and are also included in Appendix 2.

Data Analysis

Survival rates from brine exposure experiments were calculated as the proportion of individuals alive at a given time point. On occasion, individuals believed dead at one time point were found to be alive at a subsequent time

point. This was often the result of individuals being transferred into filtered site water and being given an hour to recover from treatment; if this was the case, survival rates for earlier time periods were adjusted to correct for later, higher, survival rates.

The number of dead individuals found in treatment groups may be attributed to i) individuals dead at the beginning of testing, ii) individuals that died naturally during the test, and iii) individuals that died as a result of brine exposure. To accurately report the mortality caused by brine treatment, it was necessary to exclude individuals that died from (i) and (ii) from analysis. The survival rate to brine treatment was calculated as:

$$\text{Survival rate (\%)} = TS / CS \times 100\% \qquad \text{Equation 1}$$

where TS and CS are the number of viable individuals / number of dead individuals in the treatment (15‰, 30‰, 45‰, 60‰, 77‰, 115‰) and control (filtered site water) at a given time, respectively. In cases where this equation yielded a survival rate greater than 1, this value was reduced to 1 for further analysis.

Survival rates did not follow a normal distribution (Shapiro-Wilk Normality test, $p<0.05$), and could not be markedly improved with transformation of data. Therefore, non-parametric Kruskal-Wallis tests were performed to determine if survival rates for different brine treatments or survival rates at different treatment temperatures varied significantly (Zar, 1999). Kruskal-Wallis tests were also used to determine if there was a difference in survival to brine treatment based on an individual's life history (habitat salinity, taxonomic group, collection area).

Wilcoxon rank sum tests were used to perform pair-wise comparisons of variables found to be significantly different using a Kruskal-Wallis test. For statistical analysis, any replicate, or taxonomic group within a replicate, that had less than 10 individuals was excluded. A significance level of 95% was used for all analyses.

Since non-parametric analysis allows only the examination of one variable at a time, it was often necessary to perform a separate analysis of variance for each experiment. Since these tests examined independent data, a Bonferroni correction was not needed. However, in cases where multiple tests were done to evaluate the same data, for example when differences in survival were examined between brine concentrations for all individuals in a trial and then for specific groups of organisms in a trial (see Appendix 3), a Bonferroni correction was applied when interpreting data.

Results

Zooplankton mortality was measured at six brine concentrations (115‰, 77‰, 60‰, 45‰, 30‰, 15‰) and control (filtered site water) with exposure times ranging from one hour to six days. Table 4 provides detailed sampling information and Tables 5, 6, 7, and 8 provide median survival rates for each trial evaluating brine treatment at 115‰, 77‰, 60‰, and 15‰ and 30‰, respectively. Statistical results can be found in Appendices 3 through 7.

Individuals treated with 115‰ brine for one hour had a median survival rate of 0.00% (range 0.00-5.33) (Figure 3; Table 5), and complete extermination was reached in 12 of 15 trials (Figure 4; Table 5). Only five individuals (2

unidentified copepod nauplii, 2 *Cirripedia* larvae, 1 *Nanorchestes* mite) of 13183 individuals tested were able to survive this treatment. Zooplankton exposed to 77‰ brine for one hour had a median survival rate of 0.00% (range 0.00-12.09) (Figure 3; Table 6), and complete mortality was reached in six of 15 trials (Figure 4; Table 6). A total of 126 individuals (~1.0%) were able to survive this treatment. Mortality caused by 115‰ brine was significantly higher than mortality from 77‰ brine in four experiments (Figure 4; Kruskal-Wallis, $p<0.05$; Appendix 3).

Individuals treated with 60‰ brine for one hour had a median survival rate of 0.00% (range 0.00-100.00) (Figure 3; Table 7). When this treatment was extended to two hours, the median survival rate was still 0.00%, but the range was much smaller (range 0.00-4.36) (Figure 3; Table 7). These results are not directly comparable to results from the 77‰ and 115‰ treatments above, since these results were not generated from the same experiments (see Table 4). However, there was no significant difference in survival between ballast tanks taxa exposed to brine at 60‰ and 77‰ (Figure 5c; Kruskal-Wallis, $p>0.15$; Appendix 3). A 45‰ brine exposure was much less effective than 60‰ brine treatment, as evidenced by marine taxa, collected from a 34‰ ballast tank, that were able to survive six days of exposure to 45‰ brine. At this time, the experiment was terminated although some copepods were still alive.

In Detroit River experiments, all individuals were exterminated by one hour of exposure to 30‰ or 60‰ brine treatment (322 individuals) (Figure 5a; Tables 7, 8), and these treatments are therefore considered equal (Kruskal-Wallis, $p>0.05$; Appendix 3). Three hours of 15‰ brine treatment was less effective; one

copepod nauplii and two rotifers were able to survive to give a median survival rate of 0.00% (range 0.00-29.82) (Figure 3, Table 8).

Temperature

Mortality from brine exposure was examined at 22°C and 11°C for ballast tank and Detroit River zooplankton (Figure 6). Detroit River taxa were exposed to 15‰ brine (three hours), 30‰ brine (one hour) and 60‰ brine (one hour) at these temperatures. There was no significant difference in survival between individuals tested at 22°C and 11°C (Figure 6a; Kruskal-Wallis, $p>0.3$; Appendix 4). Ballast tank taxa were also exposed to brine (60‰, 77‰ and 115‰; one hour) at 22°C and 11°C. There was no significant difference in survival between these temperatures for 77‰ or 115‰ treatment (Figure 6b; Kruskal-Wallis, $p\geq0.5$; Appendix 4). However, a treatment of 115‰ brine caused complete mortality at 22ºC, while two copepod nauplii survived this treatment at 11ºC (Table 5). It was not possible to test for a difference in survival due to temperature after exposure to 60‰ brine, because there were differences amongst experiments within treatments (see habitat salinity results; Kruskal-Wallis, $p <0.05$; Appendix 5).

Habitat Salinity

Freshwater taxa were much more susceptible to brine treatment than both brackish and marine taxa. No freshwater taxa survived one hour of exposure to 30‰ brine (Figure 5a; Table 8), whereas some brackish and marine taxa were able to survive exposure to 60‰, 77‰ and 115‰ brine (Figures 5b, 5c; Tables 5, 6, 7). In addition, marine (34‰) ballast tank taxa had significantly greater

survival after one hour of exposure to 60‰ brine than brackish-water (22‰) taxa (Kruskal-Wallis, $p<0.05$; Appendix 5). This difference was not evident after exposure to 77‰ or 115‰ brine (Figure 5b; Kruskal-Wallis, $p>0.05$; Appendix 5), but for these brine treatments, high mortality was observed in all trials (Tables 5, 6).

North Sea zooplankton was collected from the ports of Rotterdam, Antwerp and Bremen at 10 locations with salinity ranging from 1‰ to 22‰ (Table 4). There was a significant difference in survival among taxa from these locations after a one hour exposure to 77‰ brine (Figure 4; Kruskal-Wallis, $p<0.001$; Appendix 5; Wilcoxon $p<0.05$). In fact, survival was significantly greater for individuals from 20 to 22‰ habitats than for individuals from 1 to 9‰ habitats (Wilcoxon, $p<0.005$). There was no difference after one hour of exposure to 115‰ brine (Kruskal-Wallis, $p>0.05$), but at this concentration, eight of 10 trials had complete extermination and the remaining trials had very low survival rates (<0.1%) (Table 5).

Taxonomic Group

Zooplankton tested were grouped into copepods, copepod nauplii, rotifers, and "other" taxa. *Cirripedia* larvae were present in three trials (R2, R3, R5), and were considered as a separate group in these trials. "Other" taxa included mites, cladocerans (including *Bosmina*), mysids, *Leptadora*, *Diaphanasoma*, gastropods, protists, veligers, microcentipedes, insects, and *Noctiluca scintillans.* There was a significant difference in survival amongst these taxonomic groups at 77‰ and 115‰ (Figure 7; Kruskal-Wallis, $p<0.001$; Appendix 6), but no

difference at 60‰ (Kruskal-Wallis, p>0.05; Appendix 6). Four copepod nauplii, 94 *Cirripedia* larvae, and 18 "other" taxa were able to survive one hour of treatment with 77‰ brine. "Other" survivors included mites, gastropods, microcentipedes and veligers. Two copepod nauplii and three *Cirripedia* larvae were able to survive one hour of exposure to 115‰ brine. For both of these treatments (one hour exposure to 77‰ and 115‰ brine), significantly more "Cirripedia larvae" survived than copepods, copepod nauplii, or rotifers (Wilcoxon rank sum test, p<0.015), and "other" survival was not significantly different than any other group (Wilcoxon rank sum test, p>0.04).

Collection area (Port water vs. Ballast water)

Rotterdam port taxa (habitat salinity of 22‰) had significantly higher survival than ballast tank taxa (collected from 22‰) (Figure 8; Kruskal-Wallis, p<0.05; Appendix 7) after one hour of exposure to 77‰ brine. Once again, there was no significant difference when these groups were tested at 115‰ brine (Kruskal-Wallis, p>0.05; Appendix 7). At this concentration, survival of both ballast and port taxa was very low (>1.1%) (Table 5), but interestingly, survival was actually greater for ballast tank taxa (Figure 8).

Identification of Survivors

Live individuals were only preserved separately from dead individuals in North Sea trials, and as such, survivor identification was only possible for these experiments. A total of three individuals in North Sea trials were able to survive 115‰ brine treatment (Table 9). Two individuals, both collected from 21‰ water in Rotterdam, were identified as *Cirripedia* larvae. *Cirripedia* larvae were present

in a total of three trials (303 individuals), but both survivors were isolated from the same experiment. The median survival rate for these individuals after one hour of 115‰ treatment was 0.00% (range 0.00-0.09). The remaining survivor, identified as a mite of the *Nanorchestes* genus, was collected from 22% water at the port of Rotterdam. This individual was the only *Nanorchestes* mite present in trials at 115‰.

A total of 98 North Sea taxa survived one hour of 77‰ brine treatment (Table 9). These individuals were identified as 93 *Cirripedia* larvae, two unidentified "other" taxa, one *Nanorchestes* mite, one *Rhombognathides* mite, and one *Littorina neglecta*. *Cirripedia* larvae were present in three trials (381 individuals), and survivors were isolated from each. The median survival rate for these individuals was 2.06% (range 0.00-12.21). Both mites and *Littorina neglecta* were very rare in trials.

Discussion

Results indicate that NaCl brine is an effective treatment to prevent the introduction of NIS. As expected, mortality decreased as habitat salinity increased (Figure 4), and in general, greater mortality was observed at higher brine concentrations (Figure 5). A one hour, 30‰ brine treatment was sufficient to cause complete mortality in Detroit River experiments, indicating that freshwater organisms are very susceptible to brine treatment. However, brackish and marine water organisms require much higher brine concentrations (77‰ and 115‰) to reach similar mortality (>99%) after one hour of treatment.

The first objective of this study was to determine a brine treatment that would exterminate >95% of taxa in ballast tanks. One hour treatments of 77‰ and 115‰ brine were both found to be very effective (>99% mortality) against all taxa in trials (Figure 3; Tables 5, 6). Since it is most practical and cost-effective to treat ballast water with the lowest effective brine concentration, a 77‰ treatment is more desirable than a 115‰ treatment, assuming similar mortality rates for exposed organisms. In this study, a one hour, 115‰ brine treatment was statistically more effective than a one hour, 77‰ treatment in only four of 15 experiments (Figure 5). However, the 115‰ brine treatment yielded complete extermination in an additional four experiments when the 77‰ treatment did not. In this case, a biological difference exists even though no statistical difference was found. Therefore, in eight of 15 experiments, the 115‰ brine treatment was more effective, and it is considered a better ballast water treatment.

However, in addition to the brine exposure concentration, many factors can affect the salinity tolerance of a species and these factors must be examined before a treatment recommendation is made. The variables examined in this study include the temperature at application, the invertebrates' native habitat salinity, the type of invertebrate (i.e. copepod, copepod nauplii, rotifer, *Cirripedia* larvae, "other") and the location from which the invertebrate was collected (port vs. ballast tank)

The effects of salinity on taxa can be modulated by temperature (Kinne, 1963; Browne and Wanigasekera, 2000), and during the period when international ships are active on the Great Lakes, temperature can fluctuate from

0°C to 27°C (Reid and Orlova, 2002). An acceptable brine treatment must be effective throughout the shipping season, so it was necessary to consider the effect of temperature on survival in trials. Survival to brine treatment was not significantly affected by temperature at the brine concentrations examined (Figure 6), thus I would expect that brine application should be equally effective throughout the shipping season.

A species' salinity tolerance is, not surprisingly, influenced by the salinity of its habitat (Costlow *et al.*, 1966; Laughlin and Neff, 1981; Fockedey *et al.*, 2005). Consequently, it was necessary to test taxa from a variety of habitats to ensure that all taxa arriving to the Great Lakes *via* ballast water would be exterminated by brine treatment. This was accomplished by examining taxa entering the Great Lakes in exchanged ballast tanks, taxa from "high-risk" ports, and native Great Lakes' fauna. Altogether, these experiments included taxa from habitat salinities of 0‰ to 34‰. Mortality was not influenced by habitat salinity when taxa were treated with 115‰ brine, but taxa from higher salinity environments survived one hour of exposure to 77‰ brine significantly better than those from less saline environments. Specifically, significant differences in survival were found between North Sea taxa collected from oligohaline/mesohaline environments (1 to 9‰), and polyhaline (20 to 22‰) environments (Venice system, 1959; Figure 5). Treatment with 77‰ brine is therefore not recommended since it will not be equally effective for all taxa entering the Great Lakes. Nevertheless, these results are reassuring since they show that taxa entering the Great Lakes from areas with low salinity - which

would pose the greatest establishment threat to the lakes - are the least likely to survive exposure to brine treatment.

Analyzing survival by taxonomic group indicated that *Cirripedia* larvae were the group most likely to survive brine treatment at 77‰ and 115‰, and the most frequent survivor in trials. However, these individuals are not considered an invasion risk to the Great Lakes. Already, the propagule pressure for *Cirripedia* species to the Great Lakes is high, as many individuals enter *via* hull fouling. However, these individuals are marine species, and are negatively affected by freshwater exposure. A comprehensive study on hull fouling has found that *Cirripedia* are always dead or in poor condition when found attached to ship hulls in the Great Lakes (Sylvester and MacIsaac, in review). Additionally, since "other" organisms, which would include *Cirripedia* larvae, represent only 1.5% of the invertebrate organisms in ballast tanks (Duggan *et al.*, 2005), it is unlikely that they would be present in ballast in high enough densities to establish a population. Therefore, it is somewhat encouraging that *Cirripedia* larvae are the most likely taxa to survive treatment, since they pose a low risk of invasion to the Great Lakes.

Furthermore, identification of the remaining North Sea survivors confirms that most surviving taxa are unlikely to pose a risk to the Great Lakes. Altogether five species (98 individuals) from the North Sea were able to survive exposure to 77‰ brine (Table 9). These individuals included *Cirripedia* larvae, a *Nanorchestes* mite, a *Rhombognathides* mite, *Littorina neglecta*, and one unidentified species. A literature review was conducted to determine if these

individuals were likely to pose a risk of invasion to the Great Lakes. *Nanorchestes* mites are also a very low risk for invasion. These mites are typically terrestrial, but feed on algae and can be found on shores (Dr. Heather Proctor, University of Alberta, pers. comm.). Since they are a terrestrial species, these individuals would not likely survive a voyage in a ballast tank, and are therefore not expected to be able to be transported to the Great Lakes. *Rhombognathides* species are known to survive several days in high salinity water (Dr. Ilse Bartsch, German Center for Marine Biodiversity Research, pers. comm.), and could potentially survive all salinity-based ballast water treatments, including BWE. In fact, the surviving individual in trials had survived 24 hour of 77‰ brine exposure. Nonetheless, *Rhombognathides* species are not believed to be a risk for invasion, because nearly all *Rhombognathides* species are already present on the shores of Atlantic Canada and have likely had many chances to establish in the Great Lakes, thus far unsuccessfully. Finally, *Littorina neglecta* are not expected to be invasive because they are a sexually-reproducing species with very low mobility (capable of moving ~1.5m per month) (Rolán-Alvarez, 2007). With the low propagule dosage expected for "other" taxa, it is very unlikely that two individuals would survive transit in a ballast tank, be released into the Great lakes, find appropriate habitat, and be able to locate each other to reproduce and establish a population.

Two species (three individuals) were able to survive one hour of 115‰ brine treatment. These individuals were collected from polyhaline habitats of 21‰ and 22‰ in Rotterdam (Table 9), and identified as two *Cirripedia* larvae and

one *Nanorchestes* mite. As discussed above, it is very unlikely that these individuals pose an invasion risk to the Great Lakes.

Finally, this study examined the efficacy of brine treatment on taxa collected from docks/ports (Detroit River and North Sea) and ballast tanks. Taxa that have arrived to the Great Lakes in ballast water are likely in poor condition from the transit (Wonham *et al.*, 2001) and may be more susceptible to unfavourable conditions. If this is true, port taxa are expected to be more resistant to brine treatment than ballast taxa. In fact, taxa collected from port water had significantly better survival than taxa collected from ballast water when exposed to 77‰ brine (Figure 7). This pattern was not evident at 115‰, likely because this treatment is sufficiently strong to exterminate even healthy individuals. Since most experiments conducted in my study examined the survival of the more-resistant port taxa, I expect that the survival rates reported herein would be even lower in practice because all taxa would have to endure ballast water transport before treatment.

In summary, the biological evidence presented above provides support for a one hour brine treatment of 115‰ to exterminate ballast water taxa. This treatment is significantly more effective than all other treatments tested, and its efficacy is not affected by treatment temperature, species' habitat salinity, or by taxonomic group. This treatment exterminated >99.9% of individuals in trials, and the highest median survival rate in any experiment was 0.07%. Since significant differences in survival due to habitat salinity and taxonomic group were found after a 77‰ brine exposure, it is likely that these factors always affect salinity

tolerance. However, the 115‰ treatment is strong enough to overcome these effects and kill even the most resilient taxa.

In order to be recommended for use in the Great Lakes, brine treatment must comply with alternative treatment discharge standards. Given a median survival rate of 0.00% (range 0.00-5.33) after one hour of exposure to 115‰ brine, I can determine if this survival rate is indeed compliant with Canadian regulations. Regulations state that an approved alternative treatment must meet the IMO D-2 discharge standard, which requires, amongst other things, <10 viable taxa $m^{-3} \geq 50\mu m$ in discharged ballast water after treatment (IMO 2004).

Duggan *et al.* (2005) sampled 33 transoceanic ships and reported the median number of animals in residual water entering the Great Lakes from a variety of source regions. When ballast originating in the Great Lakes was excluded, a median of 280 taxa m^{-3} were found in unexchanged NOBOB ship residual water. If this water was treated with 115‰ brine, I expect that a median of 0.00 (range 0.00-14.92) individuals m^{-3} of ballast water would survive treatment. In contrast, current BWE practices result in a total abundance of 60.00 (range 0.00-5440.00) invertebrates m^{-3} remaining in a NOBOB tank or 2672.90 (range 40.00 to 26220.00) invertebrates m^{-3} for a BOB tank (Dr. Sarah Bailey, Fisheries and Oceans Canada , pers. comm.). Therefore, a 115‰ brine treatment is much more effective than BWE, and since <10 individuals are expected to be released m^{-3}, it is also in compliance with the D-2 discharge standard (IMO 2004).

Successful invasion requires propagules that can tolerate the biotic and abiotic conditions of the new habitat (Williamson, 1996; Ruiz *et al.*, 2000; Colautti and MacIsaac, 2004). Since most open ocean taxa are unlikely to establish in freshwater environments (Adolph, 1925), managers are most concerned with freshwater and brackish-water individuals. If only freshwater and brackish water animals are considered, ~50 animals are expected to enter the Great Lakes m^{-3} of ballast water discharged without treatment (Duggan *et al.*, 2005). Therefore, after one hour of treatment with 115‰ brine, I can expect 0.00 (range 0.00-2.67) freshwater and brackish water individuals m^{-3} to be released. After BWE, ships release a median of 0.00 (range 0.00-426.67) freshwater and brackish-water individuals m^{-3} of ballast water discharged (Dr. Sarah Bailey, Fisheries and Oceans Canada, pers. comm.). Therefore, the maximum density expected to be released following brine treatment would be far lower than that following BWE (e.g. 2.67 vs. up to 426.67 individuals). It is important to note that marine taxa cannot be discounted as an invasion risk, since there are several notable marine species that have established in freshwater (i.e. sea lamprey, blueback herring, alewife), however this study has shown that efficacy of brine treatment is very high even when including marine taxa in analysis.

Treatment with 115‰ brine caused 100% mortality for all zooplankton from habitats of ≤20‰, and the lowest median mortality observed in any trial was 99.93% (±0.11 SD) with individuals from a 22‰ habitat (Table 7). This trial can be used to compute the “worst-case scenario” survival rate; a combination of the highest survival rate and the highest number of individuals expected in ballast

water (2134 individuals m^{-3}). If this occurred, 1.49 (±2.35 SD) individuals m^{-3} would potentially survive to be released following brine treatment. This value is approximately six times lower than the maximum allowable discharge mandated by the IMO, and approximately 40 times lower than that reported after BWE (Dr. Sarah Bailey, Fisheries and Oceans Canada, pers. comm.).

Further, I can assess the propagule dosage expected after brine treatment. Considering that a NOBOB ship contains an average of 46.8 m^3 of residual water (Duggan *et al.*, 2005), approximately 0.00 (range 0.00-249.44) individuals will be released into the Great Lakes during deballasting after one hour of brine treatment at 115‰. This is well below the discharge standard which would allow <468 individuals. Although it is theoretically possible that 1 asexual individual can successfully found a population (Drake, 2005), propagule pressure theory dictates that the fewer individuals that are introduced, the lower the chance an invasion will succeed (e.g. MacIsaac *et al.*, 2002; Lockwood *et al.*, 2005). In all likelihood, most of the individuals in the ballast tank will be killed by exposure to brine, and those that are not may find the release habitat unfavourable or have difficulty locating mates. It is not expected that an NIS will establish with such a low propagule dosage.

Clearly, the expected number of individuals released after treatment depends on many factors and may vary greatly. However, the number of individuals released after a one-hour 115‰ brine treatment is well below the IMO's (2004) D-2 discharge standard in nearly all cases discussed here. Additionally, I am confident that the study taxa represent a sufficiently diverse

group of individuals to assume that the conclusions are robust no matter the source of zooplankton transported to the Great Lakes in ballast water.

All things considered, it is recommended that a minimum one hour treatment of 115‰ brine be used to treat ballast water in non-compliant ships entering the Great Lakes. Biological evidence provides strong support for this treatment since treatment was broadly effective and >99.9% of individuals were killed in trials (Figure 4; Tale 5), and further, analysis has shown that this treatment is compliant with the Canadian ballast water discharge regulations.

This recommendation must, however, be tempered by several caveats. First, although ballast water may contain many types of taxa, only zooplankton were tested in these experiments. Zooplankton were used as model organisms because they are abundant in ballast tanks, because their viability can be assessed easily using light microscopy, and because the Great Lakes have sustained many invasions recently by zooplankton (e.g. *Bythotrephes longimanus*, *Cercopagis pengoi*, *Daphnia lumholtzi*). Discharge standards, however, regulate not just zooplankton, but the total number of individuals for five classes of organisms (Table 1). Thus, it is necessary to consider all taxa when assessing brine treatment, and results from zooplankton alone may not reflect efficacy against all biotic groups. At a minimum, I would recommend that fish, phytoplankton, and microbes also be considered.

Since fish are a sexual species, high propagule pressure is necessary for individuals to find appropriate mates (Drake and Lodge, 2004). Although fish have been found in ballast tanks (Carlton and Geller, 1993; Wonham *et al.*,

2000), they are not expected to have high propagule pressure because they are usually excluded from ballast uptake by intake screens that prevent the entry of large animals when ballast is loaded. It is therefore expected that fish pose a low introduction risk even if they can survive ballast water treatment. Regardless, preliminary tests have shown that the round goby (*Neogobius melanostomus*), a previously introduced fish which is known to be susceptible to BWE (Ellis and MacIsaac, 2009), is killed by brine exposure of 45‰ to 60‰ (Santagata *et al.*, 2008).

Phytoplankton are constrained by the same osmoregulatory mechanisms that apply to zooplankton, but most exhibit a remarkable ability to tolerate changes in salinity (Kirst, 1989). However, this usually means that they can tolerate salinities below, rather than above, their habitat salinity (Brand, 1984). In fact, a review of 46 marine phytoplankton species reported salinity tolerances between 0 and 46‰ (Brand, 1984). Although it is not certain if exposure to 115‰ brine will kill phytoplankton, I expect that brine would negatively affect these taxa based on the information above.

Fungi, bacteria and viruses are also a concern as they can cause great problems to both human and ecosystem health. The salinity tolerance of these taxa may vary, but most fungi are killed at a NaCl concentration of two to 30‰, and excluding halophilic taxa, most bacteria are killed at a NaCl concentration of 100‰ or less (Dr. Carol Litchfield, George Mason University, personal communication). Viruses, which require a host, should be killed when host taxa

are killed. Therefore, I expect that brine treatment should be effective in eliminating most fungi, bacteria and viruses from ballast water.

Altogether, although it has not been empirically tested, I expect that all taxa that are transported in ballast water, with the exception of halophilic bacteria, will be negatively affected by brine treatment. Further, since ballast water exchange is currently relied upon to reduce the propagule pressure of all taxa, and acute tolerance to natural seawater is usually higher than NaCl (Kefford *et al.*, 2004), it is expected that brine will be at least as effective in eliminating ballast water taxa as BWE.

The next issue to consider is the environmental impact of releasing brine into the Great Lakes. Recently, there has been increasing concern about the environmental implications of road salt run-off entering waterways (d'Itri, 1992; Jones *et al.*, 1992; Forman and Alexander, 1998), and because brine would be released into the environment post-treatment, it could contribute to the problem. However, it is unlikely ships' brine would be a great concern for three reasons. First, brine would dilute readily upon its release and most aquatic invertebrates tolerate acute exposures in the doses expected (Blasius and Merritt, 2002). Second, the amount of brine entering the Great Lakes would be insignificant compared to the amount that already enters as road salt run-off each year, if this treatment is used as intended (i.e. as a backup for incomplete exchange or if a treatment technology fails) (Jenkins, 2007). Third, the net impact of treating a NOBOB ship, would be far less than that of a BOB ship that enters the Great Lakes after conducting ballast water exchange (Jenkins, 2007). I do not,

therefore, believe that brine treatment will cause a significant negative impact to the environment.

The final caveat to this study is that laboratory-based testing methods were used instead of ship-scale trials. I used lab-based studies because they are much more logistically and economically feasible, and they allowed me to manipulate variables that would not have been feasible in shipboard studies. However, since my study only examined brine efficacy *in vitro*, it is not possible to say for certain that the results would be identical to those *in vivo*. For example, my recommendation of a 115‰ treatment assumes complete mixing of brine in tanks to achieve a uniform treatment salinity. However, vertical tank mixing does not always occur when ballast water flushing is completed (United States Coast Guard, 2004). It is therefore a concern that brine may not mix thoroughly with residual waters in ballast tanks (Jenkins, 2007), and a uniform brine salinity may not be achieved. If thorough mixing does not occur, higher survival rates can be expected since lower brine concentrations are not as effective in exterminating taxa. Therefore, I recommend that ship-scale studies be conducted before treatment is put into practice.

The objectives of this thesis were to evaluate the efficacy of NaCl brine treatment to i) determine an acceptable treatment standard to exterminate >95% of ballast tank taxa, ii) determine any biotic or abiotic condition that may decrease the efficacy of this treatment, and iii) determine if mortality was consistent amongst all taxa. After thorough investigation, I believe that a one-hour treatment of 115‰ brine will exterminate nearly all ballast water taxa.

Treatment efficacy was influenced by the habitat salinity and taxonomic group of invertebrates in trials; however, at a concentration of 115‰, variation was insignificant and the treatment was highly and broadly effective. After literature review, I believe that ballast water taxa not examined in this study will be negatively affected by brine treatment. Additionally, I do not believe that brine release into the Great Lakes will be a significant hazard. However, before implementation, full ship-scale trials are necessary to ensure that similar results are seen *in vivo*. In conclusion, I believe that 115‰ brine treatment will be a very effective and beneficial treatment for ballast water that will pose little interference to commercial shipping, but greatly enhance the protection of the Great Lakes against NIS.

Table 1. Maximum density of organisms and indicator microbes discharged after ballast water treatment (Canada Shipping Act, 2006). (cfu = colony-forming unit)

Organism or Indicator Microbe	**Allowable discharge**
Organisms ≥ 50μm	<10 viable organisms m^{-3}
Organisms <50μm ≥ 10μm	<10 viable organisms mL^{-1}
Toxicogenic *Vibrio cholera* (O1 and O139)	1 cfu $100mL^{-1}$ or 1 cfu g^{-1} zooplankton samples (wet weight)
Escherichia coli	250 cfu $100mL^{-1}$
Intestinal enterococci	100cfu $100mL^{-1}$

Table 2. Major ion constituents of Natural Seawater and NaCl brine. NaCl brine was analyzed in the Metal Analysis Laboratory, GLIER, University of Windsor by J.C. Barrett.

Ion	**Natural Seawater** (g kg^{-1})	**NaCl brine** (g kg^{-1})
Sodium (Na^{+})	10.781[1]	26.343
Potassium (K^{+})	0.399[1]	0.083
Magnesium (Mg^{++})	1.284[1]	0.107
Calcium (Ca^{++})	0.412[1]	1.40
Strontium (Sr^{++})	0.008[1]	0.027
Chloride (Cl^{-})	19.353[1]	31.436
Sulfate (SO_4^{--})	2.712[1]	4.649
Bicarbonate (HCO_3^{-})	0.126[1]	Not available
Bromide (Br^{-})	0.067[1]	Not available
Boric Acid ($B(OH)_3$)	0.026[1]	Not available
Fluoride (F^{-})	0.001[1]	0.177
Iron	0.000[2]	0.001
Boron	0.004[2]	0.005

[1]Hovanec and Coshland, 2004

[2]Turekian, 1968

Table 3. Environmental data on North Sea collection locations.

Port, Country	Annual Temperature Range (°C)	Salinity range (‰)	Invasion Risk
Antwerp, Belgium	1-25	0.7-10	High[1]
Rotterdam, The Netherlands	5-25	0.2-30	High[1]
Bremen, Germany	1-24	1-24	High[1]
Great Lakes Region[2]	0-27	<0.2	N/A

[1]Ruiz and Santagata, 2007

[2]Reid and Orlova, 2002

Table 4. Zooplankton collection information, experimental treatments applied, number of replicates (reps), and number of organisms tested for each trial. Collection locations: S- BOB tank that has undergone BWE; D- Detroit River; A= Port of Antwerp; R- Port of Rotterdam; B=Port of Bremen; N- Waal River, Nijmegen.

Exp	Date	Collection			Test Temp. (ºC)	Brine Salinities Tested (‰)	Reps	# of orgs. tested
		Area	Temp. (ºC)	Salinity (‰)				
B	27/07/07	S	22.8	30	22	60, 77, 115	5	2555
1A	21/08/07	D	22.0	0	22	15, 30, 60	5	308
C	10/09/07	S	18.7	39	22	60, 77, 115	5	135
D	25/10/07	S	15.0	22	11	60, 77, 115	5	2067
E	12/11/07	S	10.0	34	11	60, 77, 115	5	1857
F	27/11/07	S	5.0	34	11	60, 77, 115	5	1443
1B	02/05/08	D	11.0	0	11	15, 30, 60	4	571
W1	16/07/08	A	20.3	4	22	77, 115	4	2083
R2	17/07/08	R	19.3	22	22	77, 115	4	2855
W2	21/07/08	A	20.5	9	22	77, 115	4	3041
R3	22/07/08	R	18.6	4	22	77, 115	4	7078
R4	22/07/08	R	17.3	21	22	77, 115	4	2025
R5	24/07/08	R	21.6	20	22	77, 115	4	7736
G1	29/07/08	B	24.0	2	22	77, 115	4	2313

W3	31/07/08	A	24.2	8	22	77, 115	4	1873
R6	01/08/08	R	21.3	3	22	77, 115	4	3740
N1	04/08/08	N	21.0	1	22	77, 115	4	1196

Table 5. Median (range) survival rate of organisms after one hour of brine exposure at a salinity of 115‰. If no range is marked, survival in all replicates is equal to that reported for the median. All taxa were collected from North Sea ports with the exception of those marked with an asterisk which were collected from BOB tanks arriving in the Great Lakes.

Exp. Temp (°C)	Source Salinity (‰)	Survival rate (%)				
		Copepod	Copepod nauplii	Rotifer	Other	All
22	1	0	0	0	0	0
22	2	0	0	0	0	0
22	3	0	0	0	0	0
22	4	0	0	0	0	0
22	4	0	0	0	0	0
22	8	0	0	0	0	0
22	9	0	0	0	0	0
22	20	0	0	0	0	0
22	21	0	N/A	0	0.09 (0-0.38)	0.07 (0-0.23)
22	22	0	N/A	0	0 (0-0.54)	0 (0-0.34)
11	22*	0	0 (0-8.29)	0	N/A	0 (0-5.33)
22	30*	0	0	N/A	0	0
11	34*	0	0	N/A	0	0
11	34*	0	0	N/A	0	0
22	39*	0	0	0	0	0

Table 6. Median (range) survival rate of organisms after one hour of brine exposure at a salinity of 77‰. If no range is marked, survival in all replicates is equal to that reported for the median. All taxa were collected from North Sea ports with the exception of those marked with an asterisk which were collected from BOB tanks arriving in the Great Lakes.

Exp. Temp (°C)	Source Salinity (‰)	Survival rate (%)				
		Copepod	Copepod nauplii	Rotifer	Other	All
22	1	0	0	0	0	0
22	2	0	0	0	0	0
22	3	0	0	0	0 (0-36.36)	0 (0-0.33)
22	4	0	0	N/A	0	0
22	4	0	0	0	0	0
22	8	0	0	0	0 (0-54.55)	0 (0-0.98)
22	9	0	0	N/A	0 (0-46.38)	0 (0-0.46)
22	20	0	N/A	0	0 (0-3.28)	0 (0-0.60)
22	21	0	0	0	0 (0-2.80)	0 (0-1.65)
22	22	0	0	0	12.21 (0-21.45)	7.75 (0-12.09)
11	22*	0	0 (0-10.22)	N/A	0	0 (0-5.78)
22	30*	0	0	N/A	66.67 (50.00-100.00)	2.43 (1.83-3.30)
11	34*	0	0	N/A	0	0

11	34*	0	0	N/A	0 (0-100.00)	0 (0-2.75)
22	39*	0	0	0	0	0

Table 7. Median (range) survival rate for organisms after exposure to 60‰ brine. If no range is marked, survival in all replicates is equal to that reported for the median. Taxa were collected from ballast tanks on BOB ships arriving in the Great Lakes, except those salinities marked with an asterisk which were collected from the Detroit River.

Collection Salinity (‰)	Exposure Time (hours)	Exp. Temp (°C)	Survival rate (%)				
			Copepod	Copepod nauplii	Rotifer	Other	All
0*	1	22	0	0	0	0	0
0*	1	11	0	0	0	0	0
22	1	11	0 (0-3.46)	2.97 (0-5.23)	N/A	0	2.37 (0-3.75)
30	2	22	0 (0-7.27)	0 (0-2.89)	N/A	0 (0-	2.24 (0-2.61)
34	1	11	0 (0-100.00)	0	0	N/A	0 (0-100.00)
34	2	11	0 (0-19.00)	0	N/A	0	0 (0-4.36)
39	1	22	0	0	0	0	0

Table 8. Median (range) survival rate of organisms collected from a salinity of 0‰ (freshwater taxa) after one hour of brine exposure at a salinity of 15‰, or 30‰. If no range is marked, survival in all replicates is equal to that reported for the median. All taxa were collected from the Detroit River.

[Brine] (‰)	Experiment temperature (°C)	Survival rate (%)				
		Copepod	Copepod nauplii	Rotifer	Other	All
15	22	0	0 (0-40.70)	0 (0-100.00)	0	0 (0-29.82)
	11	0	0	0	0	0
30	22	0	0	0	0	0
	11	0	0	0	N/A	0

Table 9. Individuals that survived brine exposure in North Sea trials.

Brine Treatment	Habitat salinity at collection (‰)	Species	Number of individuals
115‰	21	*Cirripedia* larvae	2
115‰	22	*Nanorchestes* mite	1
77‰	3	*Rhombognathides* mite	1
77‰	8	Unidentified	1
77‰	8	*Littorina neglecta*	1
77‰	9	Unidentified	1
77‰	20	*Cirripedia* larvae	8
77‰	21	*Cirripedia* larvae	15
77‰	22	*Cirripedia* larvae	70
77‰	22	*Nanorchestes* mite	1

Figure 1. Sampling sites at port of Rotterdam. Salinity (‰) at collection is indicated.

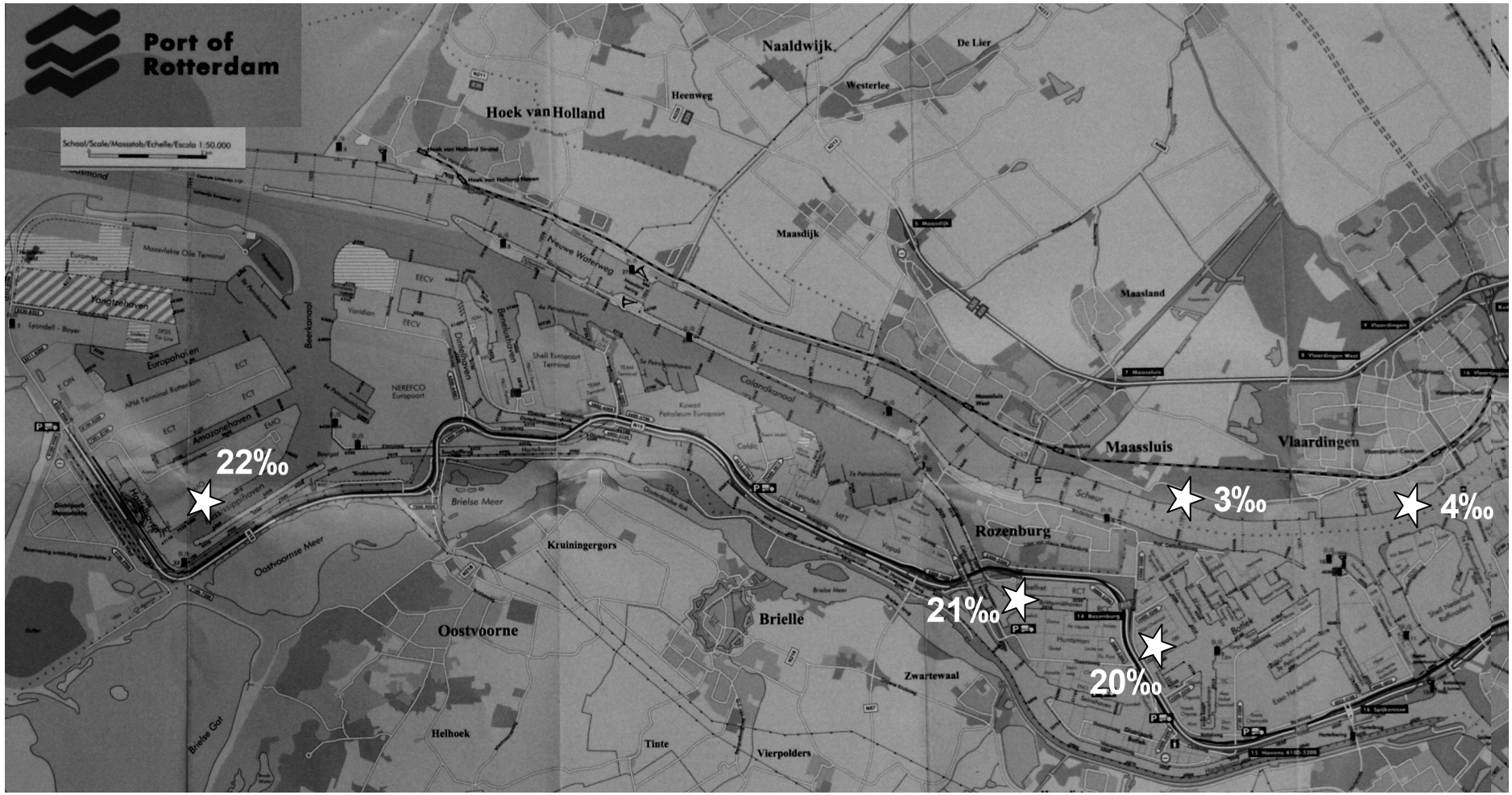


Figure 2. Sampling sites at port of Antwerp. Salinity (‰) at collection is indicated.

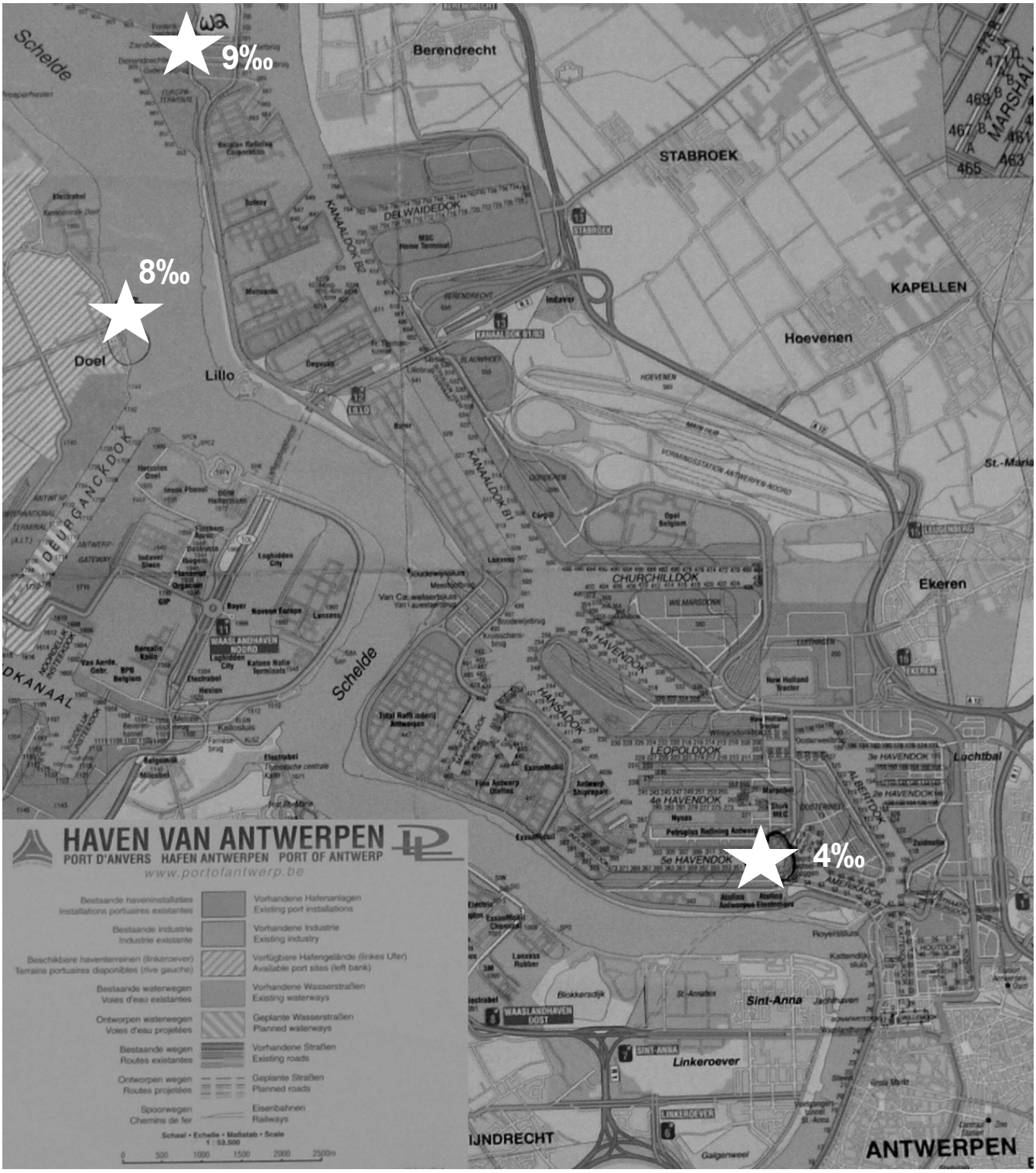


Figure 3. Mean (+SD) survival rate for zooplankton exposed to NaCl brine. White lines mark median values. Exposure time is one hour unless concentration is marked with an asterisk; (*) indicates two hours of exposure and (**) indicates three hours of exposure. Note that the first two bars (15, 30) represent data for freshwater zooplankton only, while the remaining bars represent data for brackish and marine taxa only. Survival rates have been corrected to account for survival in controls (see Methods).

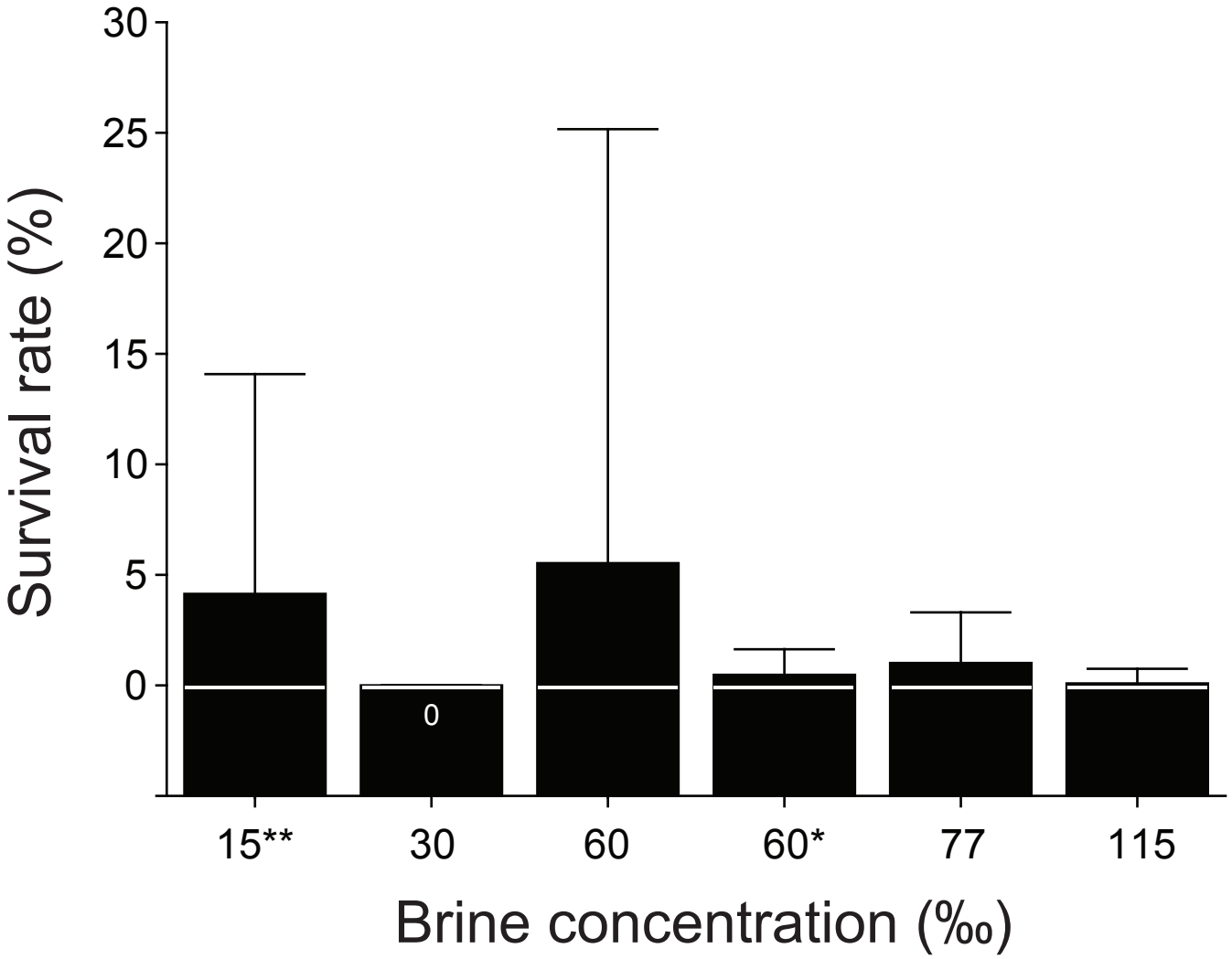


Figure 4. Mean (+SD) survival rate for zooplankton exposed to one hour of 77‰ (solid bar) or 115‰ (open bar) brine treatment. Each habitat salinity represents a separate trial. White lines (77‰) and black lines (115‰) mark median values. (*) indicates significantly lower survival in 115‰ treatment than in 77‰ treatment. Survival rates have been corrected to account for survival in controls (see Methods).

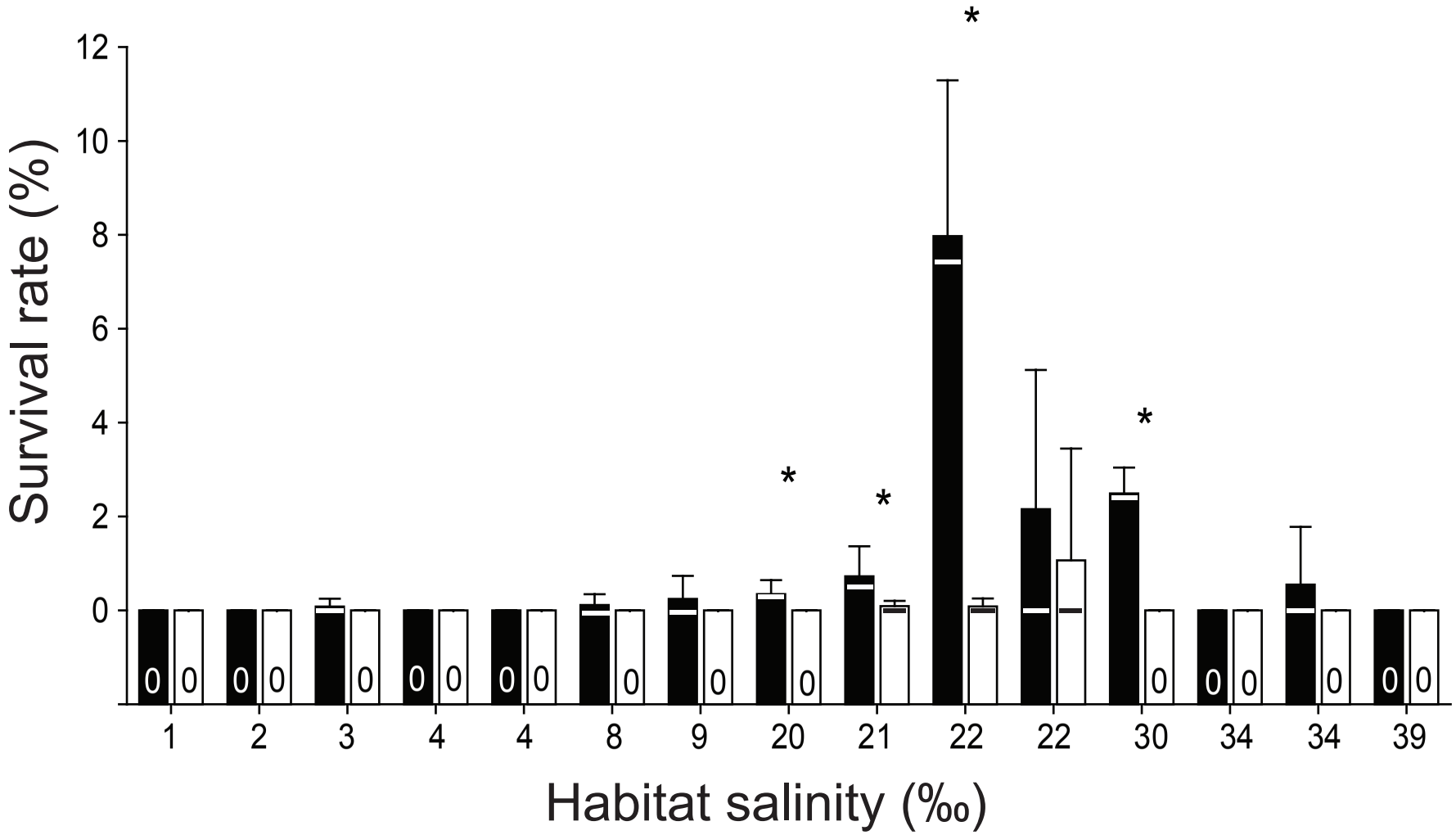


Figure 5. Mean (+SD) survival rate for (A) freshwater, (B) North Sea, and (C) ballast water zooplankton exposed to NaCl brine. White lines mark median values. Exposure time is one hour unless concentration is marked with an asterisk; (*) indicates two hours of exposure and (**) indicates three hours of exposure. Survival rates have been corrected to account for survival in controls (see Methods).

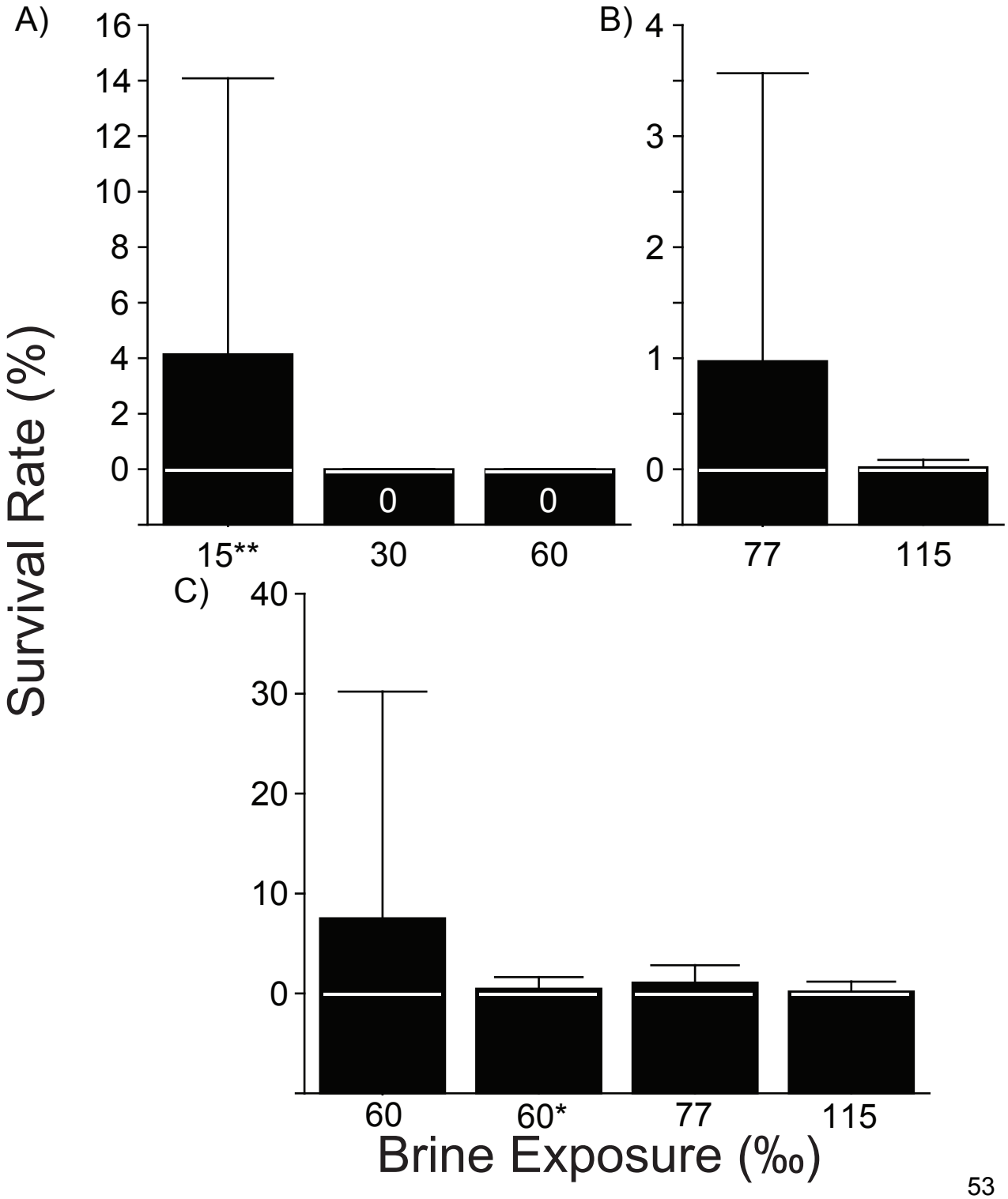


Figure 6. Mean (+SD) survival rate for (A) freshwater and (B) ballast water zooplankton exposed to brine treatment at 22°C (solid bar) and 11°C (open bar).White lines (22°C) and black lines (11°C) mark median values. Exposure time is one hour unless concentration is marked with an asterisk; (*) indicates three hours of exposure. Survival rates have been corrected to account for survival in controls (see Methods). All survival differences between exposure temperatures were found to be non-significant.

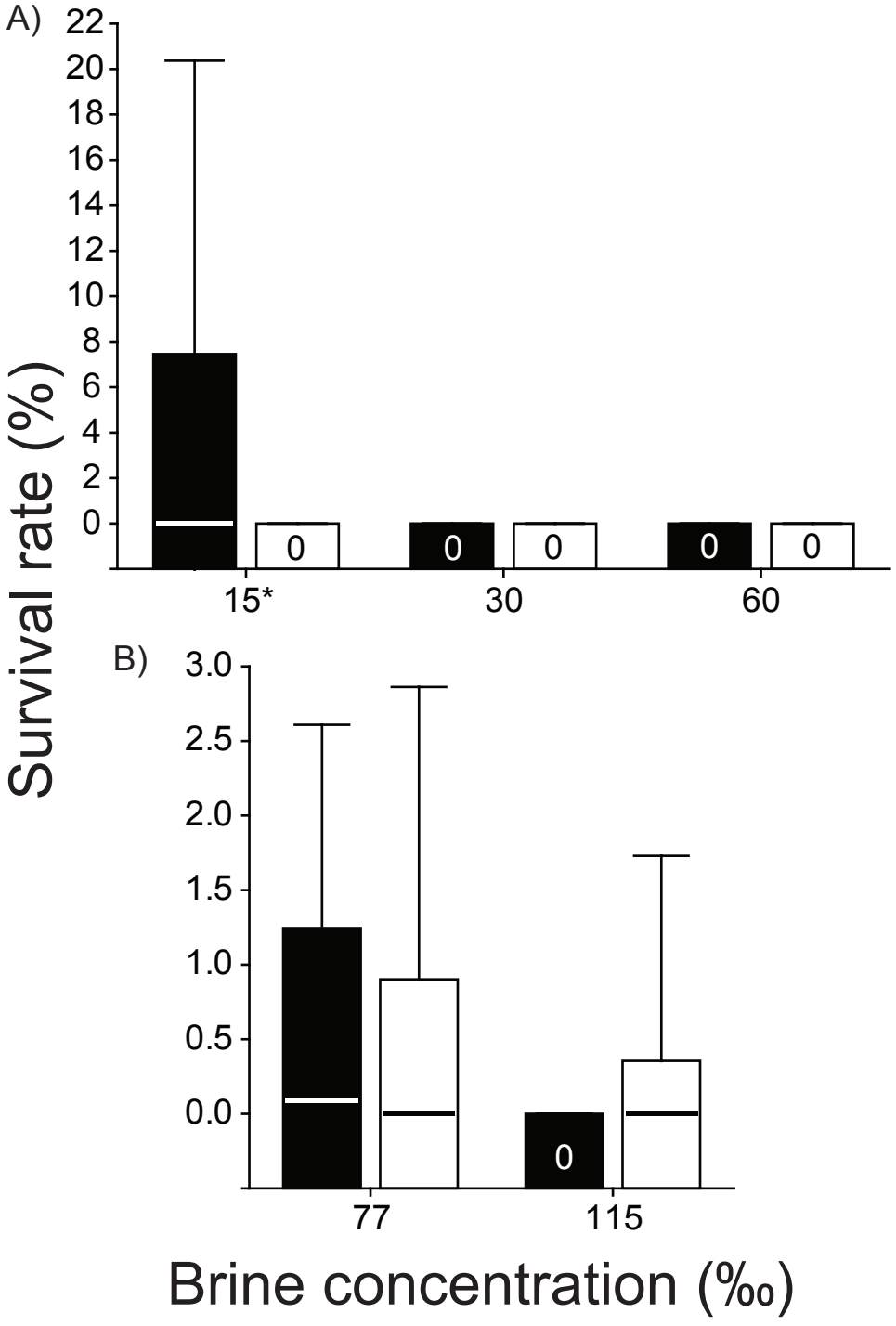


Figure 7. Mean (+SD) survival rate for copepoda (black bars), copepod nauplii (vertical stripe bars), rotifera (grey bars), "other" taxa (open bars), and *Cirripedia* larvae (diagonal stripe bars) exposed to one hour of NaCl brine. Horizontal lines mark median values. (*) indicates significant difference in survival between groups. Survival rates have been corrected to account for survival in controls (see Methods).

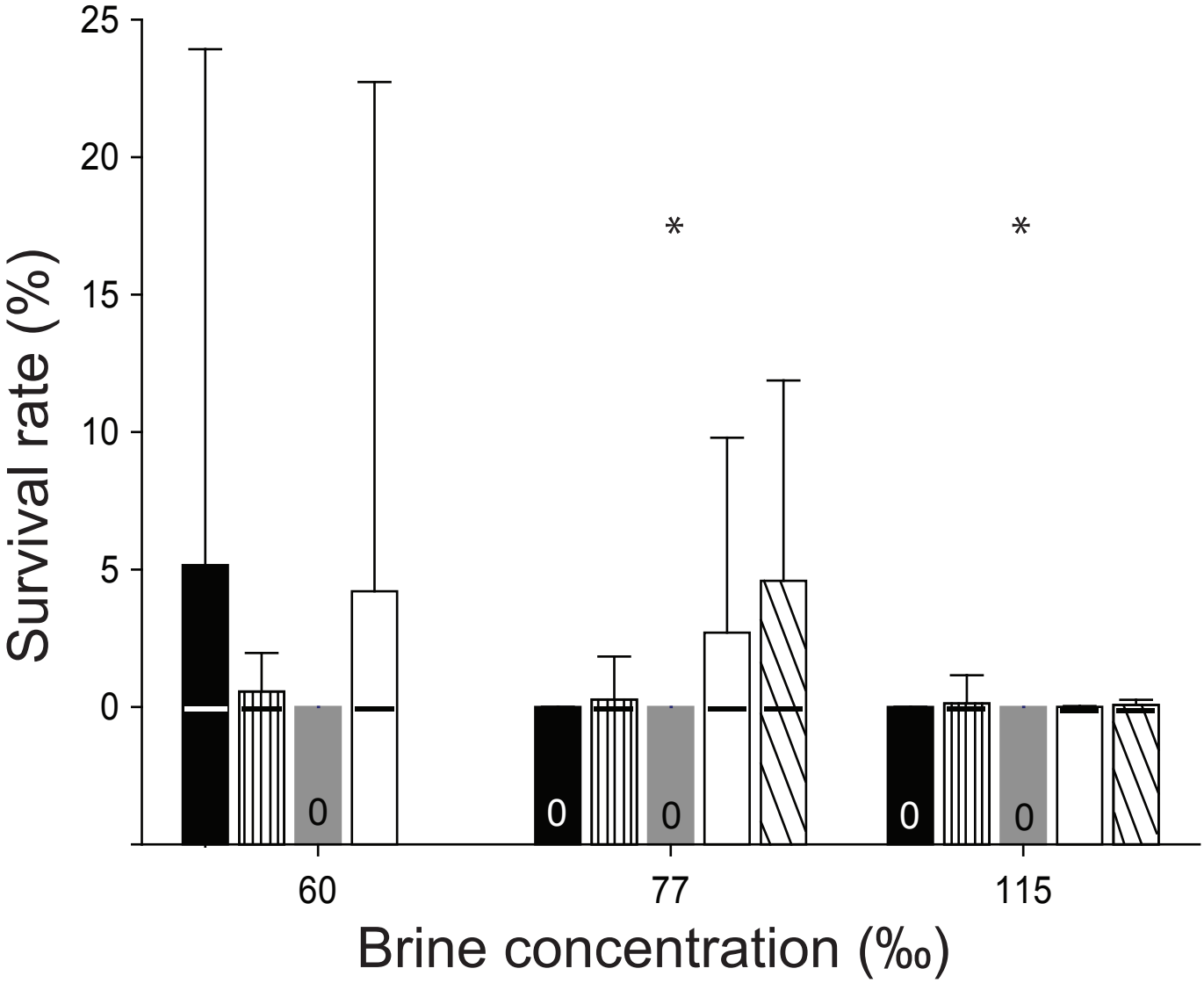


Figure 8. Mean (+SD) survival rate for zooplankton removed from ballast water (closed bars) and docks (open bars) exposed to NaCl brine for one hour. White lines (closed bars) and black lines (open bars) mark median values. (*) indicates a significant difference in survival. Survival rates have been corrected to account for survival in controls (see Methods).

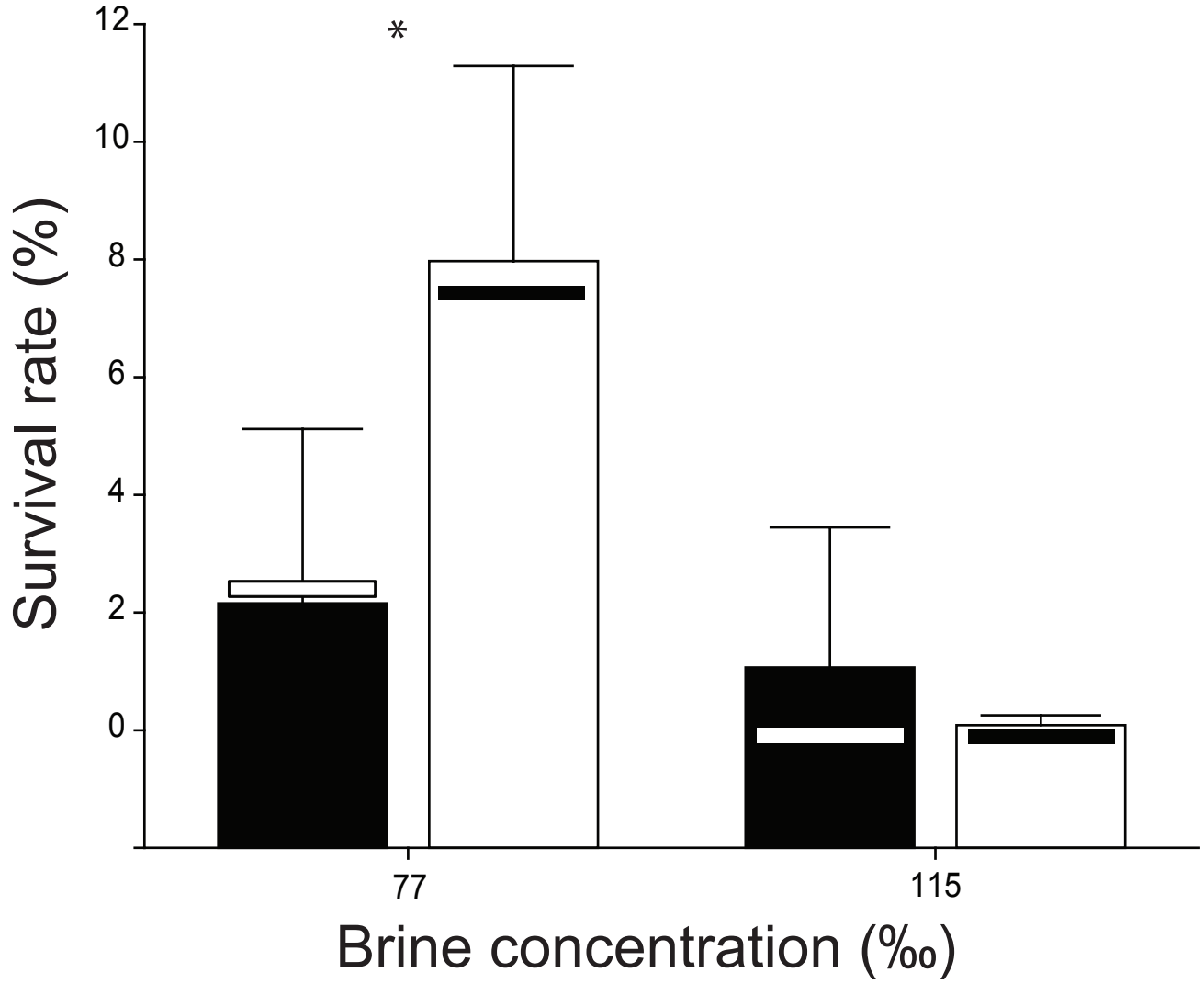
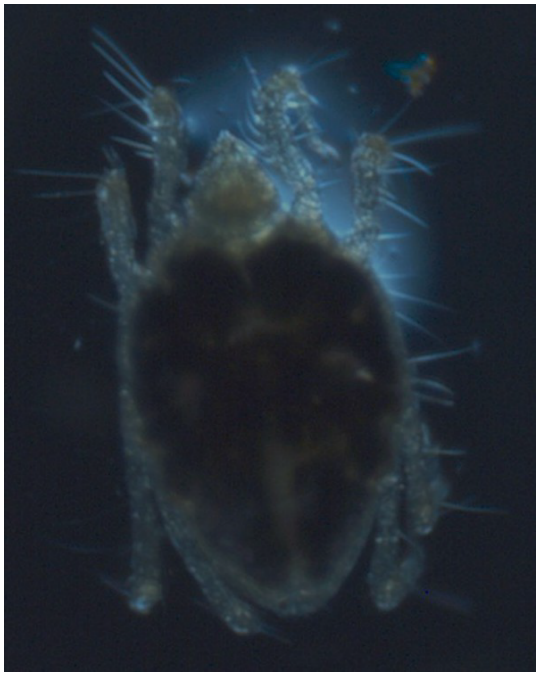
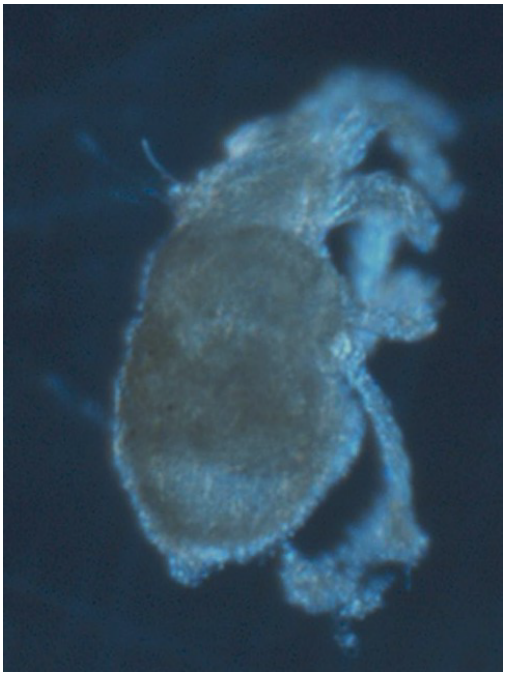


Figure 9. Photos of surviving individuals from North Sea trials. (A) *Rhombognathides* mite (B) *Nanorchestes* mite (C) *Cirripedia* larvae (D) gastropoda.

A)



B)



C)

D)

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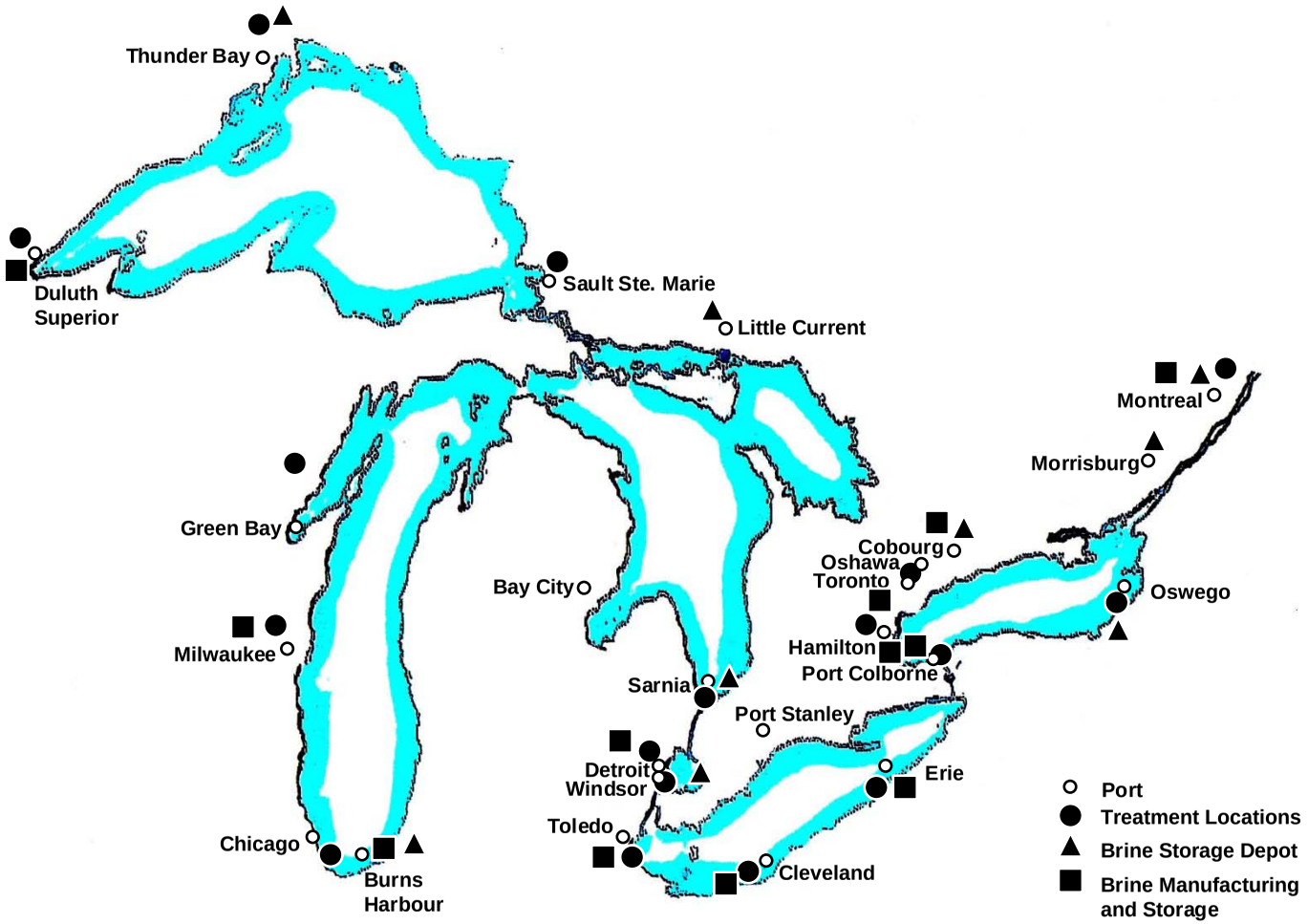
Appendix 1. Location of brine manufacturing and storage in relation to potential treatment ports (Jenkins, 2007)



Appendix 2. Non-exhaustive list of taxa included in trials.

- Annelida
  - Oligochaete
    - Oligochaete indet.
  - Polychaete
    - Polychaete larvae
    - *Phyllodocidae* indet.
    - *Spionidae* indet.

- Arthropoda
  - Arachnida
    - Acarina
      - *Nanorchestes* spp.
      - *Rhombognathides* spp.
  - Cirripedia
    - *Cirripedia* larvae
- Crustacea
  - Branchiopoda
    - Cladocera indet.
      - *Bosmina* spp.
      - *Leptodora* spp.
      - *Diaphanasoma* spp.
  - Crustacea indet. (nauplius)
  - Copepoda
    - Calanoida indet.
      - *Acartia tonsa*
      - *Eurytemora* spp.
      - *Paracalanus parvus*
      - *Pseudiaptomus coronatus*
      - *Pseudocalanus elongatus*
    - Cyclopoida indet.
      - *Cyclopina* spp.
      - *Diacyclops* spp.
      - *Halicyclops* spp.
      - *Mesocyclops spp.*
      - *Oithona helgolandicus*
      - *Oncaea borealis*
    - Harpacticoida indet.
      - *Laophonte* spp.
      - *Nitocra* spp.
      - *Diosaccus spp.*
  - Malacostraca
    - Mysidae indet.
    - Decapoda larvae
- Ctenophora
  - Nuda

- *Beroida* spp.
- Dinoflagellata
  - Noctiluciphycae
    - *Noctiluca scintillans*
- Hexapoda
  - Insecta
    - Insecta indet.
- Mollusca
  - Gastropoda indet.
    - *Littorina neglecta*
    - *Omalgyra* spp.
- Protozoa
  - Heliozoa indet.
- Rotifera
    - *Conochilus* spp.
    - *Keratella* spp.
    - *Lecane* spp.
    - *Synchaeta* spp.

Appendix 3. Results of Kruskal-Wallis test to determine the effect of brine concentration on survival. All exposure times were one hour. Significant differences are shown in bold. Collection information for each experiment can be found in Table 4. Groups: A= all organisms, C= copepoda, N=copepod nauplii, R=rotifera, L=*Cirrepdia larvae* and O=all organisms that are not copepoda, copepod nauplii, rotifera, or *Cirripedia* larvae.

Experiment	[Brine]	Group	U (Mann-Whitney) or H (Kruskal-Wallis) value	Degrees of freedom	p value
1A	30,60	A	8	1	1
1B	30,60	A	8	1	1
B	77, 115	**A**	**25**	**1**	**0.005**
		C	12.5	1	1
		N	12.5	1	1
C	60,77	A	.5	1	1
D	60,77,115	A	1.276	2	0.528
E	60,77,115	A	4.286	2	0.170
F	77,115	A	15	1	0.317
G1	77,115	A	6	1	1
N1	77,115	A	8	1	1
R2	77,115	**A**	**16**	**1**	**0.018**
		R	8	1	1
		L	**16**	**1**	**0.018**
R3	77,115	**A**	**16**	**1**	**0.018**
		R	8	1	1
		L	13	1	0.139
R4	77,115	A	8	1	1
R5	77,115	**A**	**14**	**1**	**0.047**
		R	8	1	1
		L	14	1	0.047
R6	77,115	A	10	1	0.317
W1	77,115	A	8	1	1
W2	77,115	A	10	1	0.317
W3	77,115	A	10	1	0.317

Appendix 4. Results of Kruskal-Wallis test to determine the effect of temperature on survival rates. Collection information for each experiment can be found in Table 4. Significant differences are shown in bold. Group: A= all organisms

Phase	Experiments Compared	Exposure Time and [Brine]	Group	U (Mann-Whitney) or H (Kruskal-Wallis) value	Degrees of freedom	P value
Ballast tank	BC vs. DEF	1 hr / 77ppt	A	23.0	1	0.050
	There is no significant difference between ballast water organisms exposed to one hour of 77ppt brine treatment at 22°C (BC) or 11°C (DEF).					
Ballast tank	B vs. DEF	1 hr / 115ppt	A	40	1	0.564
	There is no significant difference between ballast water organisms exposed to one hour of 115ppt brine treatment at 22°C (B) or 11°C (DEF).					
Detroit River	1A vs. 1B	3hr / 15ppt	A	U=10	1	0.317
		1h / 30ppt	A	U=8	1	1
		1h / 60ppt	A	U=8	1	1
	No significant difference in survival between freshwater organisms exposed to brine treatment at 22°C (1A) or 11°C (1B).					

Appendix 5. Results of Kruskal-Wallis test to determine the effect of habitat salinity on survival to brine treatment. All exposure times were one hour. Collection information for each experiment can be found in Table 4. Significant differences are shown in bold. Group: A= all organisms.

Experiments Compared	[Brine]	Group	U (Mann-Whitney) or H (Kruskal-Wallis) value	Degrees of freedom	P value
B vs. C	77ppt	A	5	1	0.143
	There is no significant difference in survival of zooplankton between B (30ppt) and C (39ppt) due to habitat salinity after 1 hour of exposure to 77ppt brine.				
D vs. E	60ppt	**A**	**13.5**	**1**	**0.046**
	There is a significant difference in survival of zooplankton between D (22ppt) and E (34ppt) due to habitat salinity after 1 hour of exposure to 60ppt brine.				
D vs. EF	77ppt	A	33.5	1	0.137
	There is no significant difference between D (22ppt), and E (34ppt), F (34ppt) after 1 hour of exposure to 77ppt brine.				
D vs. EF	115ppt	A	30	1	0.157
	There is no significant difference between D (22ppt), and E (34ppt), F (34ppt) after 1 hour of exposure to 115ppt brine.				
All North Sea trials	77ppt	**A**	**29.813**	**8**	**<0.001**
	There is a significant difference between Phase III experiments attributed to habitat salinity for “all” organisms.				
All North Sea trials	115ppt	A	8.211	8	0.413
	There is no significant difference between Phase III experiments attributed to habitat salinity after 1 hour of exposure to 115ppt brine.				

Appendix 6. Results of Kruskal-Wallis tests used to compare survival between grouped organisms in trials. All exposure times were one hour. Significant differences are shown in bold. Groups: A= all organisms, C= copepoda, N=copepod nauplii, R=rotifera, L=*Cirripedia* larvae and O=all organisms that are not copepoda, copepod nauplii, rotifera, or *Cirripedia* larvae.

[Brine]	Groups Compared	Kruskal-Wallis test statistic	Degrees of freedom	P value
60	C/N/R/O	3.517	3	0.319
77	**C/N/R/L/O**	**26.049**	**4**	**<0.001**
115	**C/N/R/L/O**	**35.966**	**4**	**<0.001**

Appendix 7. Results of Kruskal-Wallis test used to determine the effect of organism collection site on survival to brine treatment. All exposure times were 1 hour. Collection information for each experiment can be found in Table 4. Significant differences are shown in bold. Group: A= all organisms.

Experiments Compared	[Brine]	Group	U (Mann-Whitney) or H (Kruskal-Wallis) value	Degrees of freedom	P value
D vs. R2	**77ppt**	**A**	**2**	**1**	**0.046**
There is a significant difference in survival between port taxa and ballast tank taxa.					
D vs. R2	115ppt	A	10	1	1
There is no significant difference in survival between port taxa and ballast taxa.					

Vita Auctoris

Name:	Johanna Newbold Bradie
Place of Birth:	Windsor, Ontario, Canada
Hometown:	Amherstburg, Ontario, Canada
Education:	General Amherst High School, Amherstburg, Ontario 1999-2003, Grade 12 Diploma, Ontario Academic Credits
	University of Western Ontario, London, Ontario 2003-2007, Honours BSc Biology- Genetics and Medical Science
	Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario 2007-2009, MSc Environmental Science